University of Alberta

CD9: A Regulator of MHC class II Function

By

Adrian Mark Harbison

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

In

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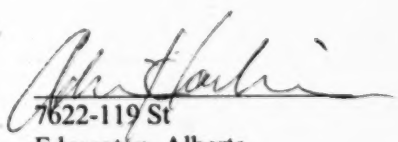

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ABSTRACT

MHC class II and CD9 are present within the triton X-100 detergent insoluble fraction (DIF) of the lymphocyte plasma membrane. Whether MHC class II associates with the actin based cytoskeleton or lipid raft components of the DIF, however, is controversial. In Raji B-cells, the proportion of MHC class II within the detergent insoluble fraction (DIF) is increased following transfection of CD9. Retention of MHC class II and CD9 within the DIF is abrogated by pretreatment of cells with latrunculin A, an inhibitor of actin polymerization. Since CD9 and MHC class II colocalize at the cell surface and coprecipitate in detergent lysates, these molecules may participate in molecular assemblies. Pretreatment with Methyl-Beta-Cyclodextrin reduced the presence of MHC class II within the DIF. Transfection of CD9 increased the endocytosis of MHC class II. Furthermore, pretreatment of cells with Methyl-Beta-Cyclodextrin was sufficient to block MHC class II uptake. Disruption of the actin cytoskeleton with cytochalasin D or latrunculin A lead to enhanced MHC class II endoytosis, suggesting that cortical actin opposes endocytosis.

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LIST OF ABBREVIATIONS

Ag	Antigen
APC	Antigen Presenting Cell
AP1/AP2	Adapter Protein 1, Adapter Protein 2
BCR	B cell Receptor for Antigen
BSA	Bovine Serum Albumin
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propane-Sulfonate
CLIP	MHC class II-associated Invariant Chain Peptide
CSB	Cytoskeletal Stabilizing Buffer
ECL	Enzyme-Linked Chromatography
FCS	Fetal Calf Serum
FITC	Fluorescein isothiocyanate
GPI	Glycosylphosphatidylinositol
HLA	Human Leukocyte Antigen
HRP	Horse Radish Peroxidase
ICAM-1	Intracellular Adhesion Molecule-1
Ig	Immunoglobulin
Ii chain	Invariant Chain
KIR	Killer Inhibitory Receptor
mAb	Monoclonal antibody

MBCD	Methyl-β-Cyclodextrin
MHC	Major Histocompatibility Complex
MIIC	MHC class II-containing Compartment
M6PR	Mannose-6-Phosphate Receptor
NK cell	Natural Killer Cell
NP-40	nonionic detergent P-40
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PFA	Paraformaldehyde
PLC	Peptide Loading Compartment
SDS	Sodium Dodecyl Sulphate
SH	Src Homology
SHP-1	SH2-containing Protein Tyrosine Phosphatase-1
SMAC	Supra-molecular Activation Complex
TAP	transporters associated with antigen processing
TCA	Trichloroacetic acid
TCR	T cell Receptor for Antigen
TRITC	Tetramethylrhodamine-5-(and-6)-isothiocyanate
VLA	Very Late Activation Antigen
VIIC	Vesicular MHC class II-containing Compartment

Chapter 1: Introduction

Section 1.1. Overview and synthesis of the hypothesis

The primary function of lymphocytes is to provide defence against invading pathogens. As such lymphocytes must house the ability to: 1) survey the tissue environment, 2) recognise foreign material as an indicator of potential danger and, 3) through tightly regulated cognitive interactions and cytokine signals co-ordinate specialized teams of effector cells to eliminate the threat. A number of key processes have been identified in lymphocytes to facilitate execution of these highly specialized tasks. The first involves processing of protein samples taken from the tissue environment into a form appropriate for communication to surveyor cells. This function is largely delegated to a subset of leukocytes that includes members of the B-cell, monocyte and dendritic lineages referred to as professional antigen presenting cells (APC). APCs require machinery uniquely suited to directing antigen presentation. Originally recognised for their role in acute graft rejection, two classes of heterodimeric surface transmembrane proteins are essential for effective antigen presentation. These are referred to as the major histocompatibility complex antigens (MHC) class I and class II. As their role in acute graft rejection suggests, MHC class I and class II antigens provide both a physical mechanism for the presentation of peptide and a source of information directing self recognition essential to immune recognition of foreign peptides.

The functions of MHC class II have been historically divided into two broad categories, exogenous antigen presentation and signalling function. The maturation and functional capabilities of MHC class II molecules are dependent upon intimate post-translational association with several distinct chaperone-like molecules. Chaperone-like molecules offer scaffolding to direct protein folding, provide trafficking signals and place restraints on the pool of peptides available for presentation. Consequently, disrupting interactions between chaperone-like proteins and MHC class II results in non-functional MHC class II that is targeted to the lysosomal compartment for destruction.

At the cell surface MHC class II antigens are capable of forming complex multi-molecular assemblages. The role of cell surface proteins in directing MHC class II functions following maturation remains poorly understood. A family of proteins that traverse the plasma membrane 4 times, referred to as tetraspanins, have recently been implicated as components of class II complexes at the cell surface and within a late endosomal compartment (MIIC; MHC class II containing late endosomal compartment) believed to function in peptide loading. Given the vital role of chaperone proteins in early MHC antigen development and the participation of surface MHC class II in macromolecular complexes it would appear reasonable that a novel set of chaperone-like proteins would regulate the function of mature MHC class II molecules at the surface and in surface-accessible peptide loading compartments. MHC class II molecules form intimate associations with members of the tetraspanin

family making them prime candidates for just such a function. CD9, a member of the tetraspanin family, is a late B-cell marker that forms physical associations with MHC class II. Here we hypothesise that the MHC class II associated tetraspan CD9 may play a pivotal role in MHC class II function.

Section 1.2 Review of the literature

1.2.1. Antigen presentation: initiating the immune response

Traditionally the role of class II major histocompatibility complex (MHC class II) in cellular physiology has been divided between two functions. The "classical function" of MHC class II involves antigen presentation to $CD4^+$ T-cells. The second involves signal transduction and activation of professional APC's following engagement of MHC class II at the plasma membrane. This second subset of activities or "non-classical function" of MHC class II has been largely studied following T-cell B-cell contact or super-antigen stimulation.

Our understanding of the immune system centres on the ability of immune cells to survey tissues at the cellular level for the production of foreign or inappropriate self-encoded protein. Recognition of peptide epitopes by both helper and effector immune cells drives the immune response and requires presentation in an appropriate context at the cell surface. The classical function of MHC class II fulfils the requirement for appropriate antigenic epitope presentation at the cell surface. MHC class II antigen

presentation is restricted to the $CD4^+$ subset of T-cells referred to as T-helper cells. By contrast MHC class I molecules are restricted to the $CD8^+$ subset of T-cells. A bifurcation in the pathway directing transport of MHC molecules to the plasma membrane and peptide-loading compartment (PLC) in addition to the presence of chaperone molecules restricts their access of MHC molecules to peptide pools. MHC class II acquires exogenous protein while traversing the endocytic pathway but is unable to access pools of endogenous antigen within the ER where MHC class I peptide loading occurs. Perhaps not surprisingly MHC class II is expressed primarily on cells with phagocytic or endocytic mechanisms for antigen uptake from the external environment. APC expressing MHC class II harbour the ability to process exogenous antigen following ingestion into the endocytic machinery. By contrast MHC class I is involved primarily in presentation of endogenous peptides processed by cytosolic proteosomes and transported through the TAP (transporters associated with antigen processing) transporter to the endoplasmic reticulum (ER) [1]. This bifurcation in antigen presentation and sorting underlies requirements for specific sorting mechanisms and/or distinct subsets of accessory molecules that influence pools of antigen available for presentation at the plasma membrane throughout the life time of MHC antigens.

The non-classical functions of MHC class II affect antigen presentation [2], direct adhesion and cytokine release [3, 4], as well as direct proliferative or apoptotic events [5-7]. The extent to which MHC class II initiates signal transduction involving phosphotyrosine remains unclear. The cellular activities invoked through MHC class II

engagement vary depending on the cell type and specificity of the stimulus [8]. Interestingly, the cytoplasmic tails of MHC class II fail to associate with any detectable tyrosine kinases [9] and are not required to initiate tyrosine phosphorylation events [10]. However, the requirement for conserved residues within the transmembrane region of MHC class II for signalling [10] does suggest the possibility that signal transduction is initiated through formation of multi-molecular complexes involving lateral associations with other integral membrane proteins. Regulation of MHC class II complex formation through cell specific or developmentally co-ordinated expression of associative proteins may alter the accessibility of MHC class II to intracellular pools of signalling molecules thereby altering the downstream consequences of MHC class II engagement.

1.2.2. Peptide Binding by MHC class II

MHC class II is expressed as a glycoprotein on the surface of APC's typically in association with bound peptide. X-ray crystallography analysis suggests that MHC class II forms a dimer of dimers [11]. Each dimer consists of non-covalently associated α- and β- subunits which generate the peptide binding region and site for T-cell receptor (TCR) binding [11]. The β-chain in addition provides conserved residues for CD4 co-stimulatory molecule recognition [12]. In addition, each dimer carries a captured peptide for antigen presentation at the cell surface within a cleft formed by a non-conserved portion of the molecule. The peptide-binding groove formed by α- and β- subunits places constraints upon the physical characteristics of peptides loaded for

presentation at the cell surface. First unlike the closed peptide-binding groove of MHC Class I, MHC Class II has an open-ended peptide-binding groove allowing it to accommodate larger peptides of 13-18 amino acid residues [11]. Secondly, the repertoire of peptides bound by a given isotype of MHC class II is further limited by the properties of non-conserved amino-acid residues lining the binding site. Unlike peptides bound within MHC Class I, MHC Class II peptides do not contain conserved anchor residues for binding within the groove nor do electrostatic interactions appear to be as crucial in determining the physical properties of peptide bound within the cleft. MHC class II appears to depend primarily upon hydrogen bonding between the backbone of 7-10 core residues of the peptide and the Class II binding groove [11, 13].

1.2.3. Peptide Acquisition by MHC class II: Classical and Alternative Peptide acquisition

Exogenous antigen enters into the APC as peptide through phagocytosis, fluid-phase endocytosis and receptor-mediated endocytosis. Ingested peptides progress through the endocytic pathway where they are progressively degraded through exposure to changing pools of acidic proteases into acceptable fragments for association with MHC class II [14]. Peptide-binding competent MHC class II molecules, however, gain access to newly processed peptide through at least two mechanisms. As a result, the studies of MHC class II peptide binding and transport to locations of peptide loading have evolved in parallel. Two chaperone-like molecules, HLA-DM and its negative regulator HLA-DO, have been identified that assist peptide-loading within the

endocytic compartment [15]. Early evidence for the presence of a distinct peptide-loading compartment (PLC) was based on immuno-gold labelling studies which identified HLA-DR1, HLA-DM and HLA-DO in compartments of the endocytic pathway containing endocytosed transferrin-neuramidase conjugates [16], BSA-gold as well as CD82 and CD63 molecules of the tetraspanin family [17]. As a result cellular transport of MHC class II within the endosomal pathway was first recognised in the late 1980's and continues to be a topic of considerable interest into the year 2000 [16, 18, 19].

The presence of MHC class II within the endosomal pathway is extensive with MHC class II being identified in a number of sub-compartments. Studies tracing the movement of endocytosed substrates and immuno-gold labelled anti-HLA antibodies have demonstrated the presence of MHC class II in early endosomes, late endosomes positive for the mannose-6-phosphate receptor (M6PR) as well as in multi-lamellar late endosomal/early lysosomal compartments comprising the MIIC [17, 20]. More importantly, it has been recognised that MHC class II gains access to the endosomal pathway through at least two separate pathways. The first route directly transports MHC class II from the ER to early endosomes [21]. The second route involves transport to the plasma membrane followed by uptake into the endocytic pathway [21].

Previously MHC Class II processing and peptide loading have been thought to occur shortly after synthesis while en route to the surface (Figure I). This pathway referred to as the "classical pathway" is dependent on protein synthesis and association of MHC

class II with Ii chain as well as with several non-classical HLA gene products [22]. Immediately following translation in the rough ER MHC class II associates with the Invariant chain (Ii) generating a nonameric complex consisting of three pairs of MHC Class II αβ-hetero-dimers associated with a preformed trimeric assembly of Ii chains [23-25]. Association of MHC class II with the invariant chain renders the immature molecule inaccessible to peptide loading within the ER where MHC class I peptide loading takes place [26]. The association of MHC class II with the Ii chain is necessary for appropriate folding of the newly synthesised MHC class II and provides sorting information in the cytoplasmic tail of Ii that allows MHC class II to access endocytic vesicles. Immature MHC class II appears to enter the endocytic pathway through early endosomes since access to MIIC and multi-vesicular bodies preceding transport to the cell surface is dependent on early endosomal transport [27].

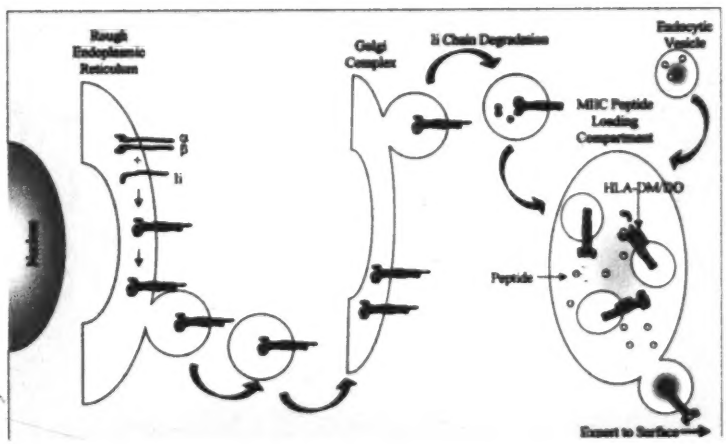

Figure I. *MHC class II maturation and restricted antigen presentation.* MHC class II α and β chains are translated into the ER where they assemble via Ii into a nonameric complex. MHC class II peptide loading takes place in the late endocytic compartment following Ii chain dependent delivery to the endosomal compartment. Degradation of the Ii chain occurs en route to the MIIC compartment. HLA-DM and HLA-DO are thought to mediate CLIP exchange for processed peptide within the MIIC compartment.

As mentioned previously, the MIIC is thought to act as the site corresponding to class II-associated invariant chain peptide (CLIP) exchange for processed exogenous peptide. Peptide-loading within the late endosomal compartment is facilitated by two non-classical HLA gene products HLA-DM and HLA-DO [15, 28-30]. Together these two chaperone-like proteins facilitate peptide loading and editing of unsuitably matched peptide-MHC class II complexes (Figure II)[17, 31, 32]. MHC class II molecules that fail to exchange CLIP for processed peptide are directed to lysosomes for degradation. This route of MHC Class II maturation and peptide loading emphasises the requirement of accessory proteins in normal class II function.

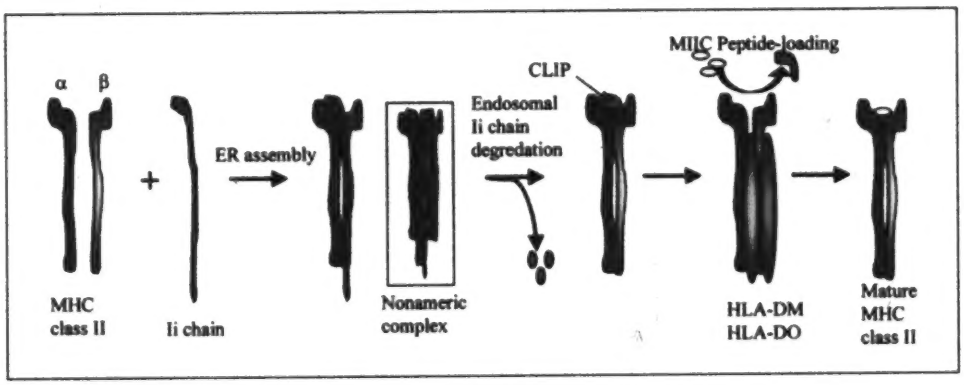

Figure II. *Association of MHC class II with non-classical HLA gene products during de novo synthesis and peptide loading.* Following MHC class II translation into the rough ER each α– and β– chain associates with a single copy of the invariant chain which then trimerizes into a nonameric complex through signals located in the Ii chain. Association of MHC class II heterodimers with the invariant chain complex provides the necessary targeting signals for delivery to the early endosomal compartment. During transport through the endosomal compartment acidic proteases are thought to progressively digest the Ii chain leaving only the class II-associated invariant chain peptide bound to the MHC class II heterodimers. Within the late endocytic/early lysosomal compartment, HLA-DM and its negative regulator HLA-DO catalyze exchange of CLIP for processed antigen.

Vesicular trafficking from the plasma membrane delivers immature complexes to the early endosomal compartment and appears to involve signals within the cytoplasmic tails of MHC class II heterodimers and the Ii chain. Following entrance into the early endosomal compartment immature Ii-MHC class II complexes migrate to the late MIIC compartment, as described for the classical pathway, for peptide loading. Identification of the alternate pathway for MHC class II antigen acquisition is compatible with a role for surface proteins in the regulation of MHC class II trafficking.

More recently a third novel route of MHC class II peptide acquisition has been identified that involves recycling peptide-experienced MHC class II. The recycling pool of mature MHC class II is thought to exchange peptide within an early endosomal compartment (VIIC; vesicular MHC class II compartment) followed by rapid transport back to the surface [1, 10]. The pathway directing mature MHC class II through a recycling component of the endocytic compartment acts independently of Ii chain by directing MHC class II to early endosomes and results in acquisition of relatively unprocessed peptide. Experiments utilising chemically cross-linked antigens suggest that recycling MHC class II presents a rapidly degraded subset of peptide providing the opportunity for a rapid immune response to exogenously added antigen [22, 33]. Interestingly, recent studies have identified MHC class II bound to full-length proteins in both human and murine systems pointing to a mechanism for the loading of relatively unprocessed antigen [34]. Furthermore, the pool of internalizing and recycling MHC class II molecules reaches equilibrium within 15min [22] which

supports rapid loading required to meet the demands of the process described above. Curiously, the stability of MHC class I peptide-loading is optimal at temperatures below 37^0C whereas MHC class II stability is optimal at physiological temperatures [35]. This observation suggests that the thermal stability of empty MHC class II molecules may be substantially greater than MHC class I and may directly influence the propensity of recycling MHC class II to exchange peptide within the endocytic pathway.

A portion of Ii and HLA-DM are delivered to the cell surface where they regain contact with MHC class II. After regaining access to MHC class II at the surface HLA-DM and Ii could potentially influence release of peptide from MHC class II and return mature class II to the late endosomal PLC. In support of this model, HLA-DM is thought to perform an editing function within the late endosomal PLC compartment. HLA-DM editing function is thought to proceed through separation of αβ-subunits causing MHC class II structure destabilization resulting in accelerated peptide release and re-loading. Interestingly, a subset of 10-20% of all HLA-DM molecules are present on the surface of B-lymphocytes where they are engaged in complexes containing empty HLA-DR molecules. MHC class II within these complexes show increased rates of peptide loading [15, 22, 30] suggesting that HLA-DM and Ii may direct a novel peptide exchange pathway.

1.2.4. Sorting of MHC class II

Two common motifs involved in receptor internalisation and targeting to endosomal compartments can broadly contain a tyrosine-based motif (YXXI; Y=tyrosine, X=any amino acid, I=any bulky hydrophobic amino acid) present in HLA-DM or leucine-based motif as seen in HLA-DR and Ii chain [36]. Vesicle budding from the plasma membrane can be initiated through clathrin-coated pit formation. Clathrin, when targeted by adaptor proteins to the plasma membrane assembles into a spherical scaffold that assists in invagination and pinching-off of newly forming vesicles. Either of the sorting motifs described above can initiate clathrin-mediated coated-pit formation as AP1, AP2, and AP3 adaptor complexes form direct interactions with these residues through their medium chain subunits [37-39]. Shu-Hui *et al.* using a dominant negative form of clathrin, termed hub fragments, that have been shown to inhibit clathrin mediated internalisation of transferrin cause cell surface accumulation of both HLA-DR and HLA-DM in HeLa 229 cells [40]. This observation suggests that MHC class II internalization from the plasma membrane involves clathrin coated-pit assembly. However, the regulatory mechanism directing MHC class II internalisation from the plasma membrane remains controversial.

The sorting of MHC class II appears to be influenced by at least two distinct signals. The first is located in the short cytoplasmic tail of the MHC class II beta-chain. In polarized Madin-Darby canine kidney cells, phenylalanine-leucine residues within the cytoplasmic tail of the beta-chain are required for efficient internalization and

localization of MHC class II molecules to basolateral early endosomes. Sorting to apically located, late multi-vesicular endosomes (MIIC) does not depend on signals in the class II cytoplasmic tail as both wild-type class II molecules and mutant molecules lacking the phenylalanine-leucine motif were found in these compartments [41]. Similarly studying T-cell activation, Pinet *et al.* demonstrated that truncation of either the alpha or beta cytoplasmic tail nearly eliminates internalization of MHC class II molecules and presentation of inactive virus particles [42]. The invariant chain-dependent presentation of matrix antigen from the same virus particles is unaffected by these truncations. Thus, access of MHC class II to a recycling early endosomal PLC compartment is sensitive to the presence of sorting signals within the cytoplasmic tail of the MHC class II beta-chain whereas access to the conventional MIIC PLC compartment is independent of MHC class II encoded signals.

The second sorting signal to direct MHC class II trafficking is provided by the cytoplasmic tail of the Ii chain. Ii chain association with MHC class II heterodimers influences newly synthesised MHC class II sorting and to a lesser extent the mature surface population MHC class II. MHC class II association with the Ii chain is dependent upon both the hydrophobic transmembrane domain of the Ii chain [23] and subsequent insertion of the CLIP fragment into the peptide-binding domain of class II molecules. Two distinct regions of the 30 amino acid residue cytoplasmic tail of Ii have been identified as endosomal targeting signals. A leucine-isoleucine (LI) motif within one region of the cytoplasmic tail and a methionine-leucine (ML) motif in a second region both of which are essential for efficient internalization [43]. Of the two

sorting signals, the LI motif appears to be particularly potent. X-ray crystallography studies undertaken by Motta *et al.* indicate that leucine signals present in the Ii chain are not continuous and exist instead as "signal patches" within a 3-dimensional structure [44]. Some controversy still exists as to whether or not the signals present within the beta-chain of MHC class II molecules are sufficient to direct recycling pools of MHC class II into early endosomal MHC class II containing vesicles (CIIV). Recent studies by Moller *et al.* have addressed this issue using a series of HT-29 variant cells line differentially expressing MHC class II and Ii. These studies indicate that:

> (i) the majority of Ii molecules physically associate on the cell plasma membrane with class II dimers to form DR alpha beta:Ii complexes; (ii) the presence of surface Ii is a prerequisite for the rapid uptake of HLA-DR-specific monoclonal antibodies into early endosomes because only the surface DR^+/Ii^+ phenotype, and not the DR^+/Ii^- variant, efficiently internalizes; and (iii) the HLA-DR:Ii complexes are targeted to early endosomes, as indicated by co-localization with the GTPase, Rab5, and endocytosed bovine serum albumin [45].

1.2.5. Association of MHC class II with the Cortical Actin Cytoskeleton

Association of MHC class II with the cortical cytoskeleton was described as early as 1988 when Newell *et al.* co-purified murine MHC class II with actin [46]. Recognition of cortical actin cytoskeleton involvement in MHC class II function developed further

in 1991 when St. Pierre *et al.* demonstrated that MHC class II signalling required cytoskeletal rearrangement during Ag presentation in accessory signal-dependent hybrids [47]. Since this time a number of groups have investigated the impact of actin polymerization inhibition on MHC class II function. Kinch *et al.* in 1993 provided evidence that CD4/MHC class II directed T-cell/B-cell conjugates could be dissociated by disruption of the cortical actin cytoskeleton with an inhibitor of actin polymerization, cytochalasin D [48]. Actin polymerization also influences delivery of newly synthesised MHC class II molecules to late endosomal compartments as treatment of a B-cell with cytochalasin D delayed degradation of the Ii chain [49].

Productive T-cell/B-cell interaction is a process requiring prolonged interaction between APC and helper T-cell. During cognitive interaction, successful induction of cell proliferation and activation relies upon the ability to retain receptors within a defined region of the membrane. Disrupting mechanisms that constrain lateral diffusion may potentially interfere with activation. Carboxy truncated MHC class II molecules display increased lateral diffusion within the plasma membrane thought to correlate with loss of anchorage to the underlying cytoskeletal matrix [50] and are inefficient in antigen presentation. The inability to generate signals that lead to interaction with the cytoskeleton is thought to block activation [51].

Whether direct interaction with the actin matrix is possible has been questioned due to the short cytoplasmic domain of MHC class II α and β-chains. Study of cytoplasmic tail deficient MHC class II α,β-heterodimers by Chin *et.al* indicate that both the

cytoplasmic and transmembrane domains contribute to class II interaction with the cytoskeleton [52]. How association with actin cytoskeleton is regulated, however, remains controversial. Crosslinking MHC class II on the cell surface translocates MHC class II into the nonionic detergent P-40 (NP-40) detergent insoluble fraction indicating that MHC class II association with the actin cytoskeleton may be dynamically regulated [53]. Association of MHC class II with the actin cytoskeleton may be further complicated by interactions with other cytoskeletal associated proteins within the context of supra-molecular complexes. In T-lymphocytes, antibody induced crosslinking of the tetraspanin molecule CD82 induces its association with the cortical actin cytoskeleton. Furthermore, disruption of actin assembly with cytochalasin D blocks the co-stimulatory function of CD82 [54, 55]. In other lymphocyte cell lines, MHC class II has been shown to associate with a network of tetraspan proteins including CD82 [56]. It is possible then that other cell surface proteins may be involved in anchoring MHC class II to the cortical actin cytoskeleton.

Despite these observations, the ability of MHC class II to directly associate with the cytoskeleton remains controversial because traditional cellular fractionation techniques failed to differentiate between the actin cytoskeleton and components of lipid raft. Triton X-100 and NP-40 detergent extraction procedures utilized for the purification of polymerized actin and actin-binding proteins [57] retained both components within the detergent insoluble material. More recently, cellular fractionation techniques using sucrose density-gradient ultra-centrifugation have been used to further differentiate

between the low-density lipid raft and high-density actin cytoskeleton components of the detergent insoluble fraction [58].

1.2.6. MHC class II signalling, organization of SMAC's and Lipid rafts

The role of MHC class II in cellular response to pathogenic insult and immune system development are not limited to shuttling of processed antigen from specialized cytosolic compartments to the surface for presentation but extend to the delivery of dynamic intracellular signals. The involvement of MHC class II in B-cell activation and delivery of dynamic intracellular signalling has been referred to in the literature as the "non-classical" function of MHC class II. Studies aimed at understanding the downstream effects of MHC class II ligation and clustering with anti-MHC class II monoclonal antibodies and super-antigen date back to the early 1990's. Over the following decade, a broad range of studies have implicated MHC class II signalling in the regulation of both cytosolic and nuclear responses to class II engagement [59]. MHC class II signals are thought to influence cell adhesion, modulation of the antigenic pool for presentation, regulation of proliferation and nuclear factor activation, cytokine production and release, apoptotic cell death, and homotypic aggregation [6, 8, 60, 61]. Emerging from these studies has been the recognition that MHC class II regulation of cellular responses is both cell type specific and in many cases dependent on the stage of APC development during hematopoiesis [8]. Understanding the downstream effects of MHC class II signalling will require identification of the function of MHC class II-associated proteins at the plasma

membrane. As mentioned previously, MHC class II at the cell surface is found within large multi-molecular complexes containing cell lineage dependent markers such as CD19 as well as members of the tetraspanin family [53, 56, 62-64].

Research into lymphocyte receptor clustering and activation has focused on T-cell signalling through the T-cell Receptor (TCR) complex. Boniface *et al.* have provided evidence that T-cell activation following formation of a T-cell/B-cell interface requires multivalent TCR-MHC interaction [65]. Similarly, Grakoui et al. have proposed that the determinative event in productive TCR signalling involves formation of a stable central cluster of TCR within the region of lymphocyte contact, which they refer to as the immunological synapse [66]. A TCR clustering requirement in activation suggests that mechanisms for lateral organisation of receptors within the plasma membrane likely exist. Previous investigations into TCR clustering have identified two possible mechanisms directing organisation of the immunological synapse or supra-molecular activation complex (SMAC)[66-68]. The first involves directional transport of surface receptors anchored to the cortical actin cytoskeleton. Wulfing and Davis (1998) have demonstrated that co-stimulation leads to enhanced and sustained intracellular signalling resulting in directional transport of anti-ICAM-1 coated beads to a point of cell-cell contact [69]. Furthermore, they identify actin cytoskeleton and myosin-based motors in directed transport of ICAM-1 to the site of cell-cell contact. The second involves segregation of molecules based upon differential miscibility in the lipid bilayer into lipid raft [58, 70-72].

Traditional theory of plasma membrane organization viewed the lipid bilayer as a relatively homogeneous exterior allowing equal access of proteins to all regions of the cell. More recent observations suggest a more heterogeneous model of plasma membrane structure in which laterally segregated regions of bilayer components are maintained. Lipid raft represents one mechanism by which lateral segregation of bilayer constituents could be mediated. Originally identified as a low buoyant density component of the Triton X-100 detergent insoluble fraction, lipid rafts are enriched in glycososphingolipid, GPI-anchored proteins, cholesterol and distinct signalling molecules including members of the Src family of tyrosine kinases [71]. The role of lipid raft lymphocyte activation has been primarily studied in T-cells. In human T-cells CD28 co-stimulation results in polarization of GM1 glycosphingolipid, a marker of lipid raft, to the site of TCR engagement [73]. Whether lipid rafts primarily direct lateral segregation of receptors or whether they additionally provide points of multi-molecular signalling complex assembly remains controversial. Recruitment of lipid raft to a site of TCR engagement results in increased and prolonged tyrosine phosphorylation and Lck consumption [73]. Similarly, accumulation of actin underlying lipid rafts is dependent upon phosphotyrosine signalling and may involve both Lck and Fyn tyrosine kinases in Jurkat cells [58]. These observations suggest that lateral organization of receptor and signalling complexes by lipid rafts may increase the efficacy of TCR engagement. Studies into NK cell signalling have pointed to both positive and negative regulatory signals directing reorganization of lipid rafts suggesting that regulation of raft distribution may greatly influence cell signalling from the plasma membrane. In these studies, MHC-bearing tumor targets were able to

block raft redistribution. This inhibition was dependent on the catalytic activity of killer inhibitory receptor (KIR)-associated SHP-1, a Src homology 2 (SH2) domain containing tyrosine phosphatase. Raft redistribution may therefore critically influence cellular responses such as cell-mediated cytotoxicity [74].

Several lines of evidence indicate that lateral segregation of proteins in the plasma membrane may precede B-cell activation following T-cell/B-cell contact. Recently, Huby *et al.* have provided evidence that intracellular phosphorylation following MHC class II engagement on the surface of a monocyte line actually requires co-aggregation of lipid rafts and an associated tyrosine kinase activity [75]. Lateral organization of the lipid environment may alter the local signalling environment of MHC class II complexes similar to immunological synapse organization observed in T-cells. Interestingly, actin may be involved in antibody induced patching and capping, since fodrin and F-actin accumulation under caps can be inhibited by cytochalasin D [76].

1.2.7. CD9 association with Lipid rafts and MHC Class II

As mentioned previously, MHC class II can associate with a number of proteins both on the surface and within the endocytic compartment of antigen presenting cells. Of particular interest are a family of nearly 20 related proteins identified as having four transmembrane domains referred to as the tetraspanin family. Tetraspanins share structural features with ligand-gated ion channels despite a lack of any sequence homology [77]. Conservation of sequence between tetraspanin family members is

generally restricted to the transmembrane region that typically contains a few highly conserved polar amino acids [77]. Tetraspanins are known to associate with signalling molecules and participate in cell adhesion, cell differentiation and activation [54, 77]. The mechanism by which tetraspanins effect cellular signalling may relate to their participation in and potential role in formation of protein complexes [56, 77, 78]. Because of their influence in stabilization of signalling complexes, tetraspanins have been called molecular facilitators [56]. The activities of individual tetraspanins have yet to be identified as many members display pleiotropic and apparently redundant activities. This is further complicated by expression of multiple family members within a given cell type. The distribution of tetraspanin expression is ubiquitous for some members (CD63, CD81, and CD82) while in others, it tissue specific (CD53, CD37). Furthermore, expression may be developmentally regulated. In B-lympocytes, CD9 is only expressed by immature and activated cells whereas CD37 is found only on mature B-cells [79].

Two tetraspanins have been identified within the detergent insoluble plasma membrane. Utilizing the high sensitivity of nanoelectrospray tandem mass spectrometry Shevchenko et. al. identified CD9 and CD81 [80] in addition to two proteins of the p23/p24 family of putative vesicular trafficking receptors and caveolin-2 within the detergent insoluble fraction. Crosslinking human leukocyte antigen (HLA-DR) on the surface of THP-1 cells induces its entry into the glycolipid-enriched membrane fraction of the plasma membrane [75] implicating MHC class II as a potential component of the lipid raft. As discussed above, lipid rafts may alter the

signaling environment of MHC class II by limiting access to pools of intracellular signaling molecules or providing anchorage sites to the cortical cytoskeleton by regulating actin remodeling. Lipid raft may also directly affect MHC class II function through regulation of vesicular trafficking and sorting. In polarized cells, loss of apical and basolateral sorting has been attributed to disruption of lipid raft by removal of cholesterol with methyl-beta-cyclodextrin (MBCD)[81-83]. Whether or not lipid raft access or tetraspanin expression modifies MHC class II signaling or trafficking is unknown. However each could fundamentally affect the outcome of MHC class II engagement through regulation of downstream effector function.

MHC class II has been shown to associate with a number of tetraspanin family members including CD9, CD63, CD81 and CD82 [56, 63, 64, 84, 85]. Association of MHC class II with CD9, CD81 and CD82 may link it to co-stimulatory molecules such as CD19 [54, 86]. The threshold for B-cell receptor (BCR) signalling is lowered through formation of a complex that involves CD19, CD21, and Leu^{-13} [87, 88]. Given the involvement of tetraspanins in BCR and integrin mediated signals it is possible connecting MHC class II to the tetraspan web may influence both signalling function and trafficking.

Whether or not expression of developmentally regulated tetraspanins affect MHC class II function remains unanswered but could play a pivotal role in B-cell selection and development. CD9 is expressed only on developing and activated mature B-cells where it is associated on the surface with MHC class II [56]. Activation and homotypic

aggregation events described in platelets are influenced by CD9 induced signalling events [89]. Anti-CD9 monoclonal antibody-induced platelet activation has been attributed to both the classical phosphoinositide turnover and synthesis of 3-position phosphorylated inositol phospholipids [90] as well as Ca^{2+} flux induction [91] and tyrosine phosphorylation induced by p72syk [89]. Seehafer *et al.* have suggested that initiation of downstream signaling events generated by CD9 antibody ligation may be a result of association with a select pool of small G-proteins [92]. In pre-B-cells, CD9 has been identified as a tetraspan involved regulation of membrane adhesion and motility [79, 93, 94]. Transfection of CD9 into the mature B-cell line Raji is sufficient to increase adhesion on laminin and fibronectin mediated through late expressed integrins VLA-4 and VLA-5 [79, 94, 95]. The involvement of CD9 in membrane adhesion and fusion is supported by recent studies involving CD9 knock-out mice that implicate CD9 in egg and sperm fusion. $CD9^{-/-}$ knockout females present with severely impaired fertility as a result of impaired fusion mediated through CD9-associated integrin $\alpha 6\beta 1$ [96-98]. CD9 and CD81 also appear to generate signals promoting muscle cell fusion and support myotube maintenance in murine C2C12 myoblast cells [99]. As such, developmental modulation of tetraspanin expression may alter cellular responses to external stimuli in lymphocytes.

Chapter 2: Materials and Methods

Section 2.1. Reagents and Antibodies

Cell culture products were purchased from GIBCO (Burlington, On). Methyl-beta-cyclodextrin (MBCD), cytochalasin D and Phalloidin-FITC were purchased from Sigma (Oakville, On). Transferrin-FITC and latrunculin A were obtained from Molecular Probes (Eugene, Or). Rabbit polyclonal anti-MHC Class II antibodies were a gift from Dr. Michael Langender and monoclonal antibody 7H.3 was the gift of Dr. Hidde Ploegh at Biomira (Edmonton, Ab.). Monoclonal antibodies against CD9 were generated as described previously by our lab. Anti-β1 integrin mAb AP1.138 also was obtained from Dr. Langender. Affinity-purified rabbit anti-mouse heavy and light chain immunoglobulin, Goat anti-rabbit Ig and horseradish peroxidase conjugated Goat anti-mouse Fc specific immunoglobulin were purchased from Jackson Immunochemichals. All other reagents were purchased from Sigma (Oakville, Ont.).

Direct fluorescent conjugates of anti-MHC Class II (mAb 7H.3-Alexa 594 and mAb 7H.3-Alexa 488), 7H.3 Fab fragments and anti-CD9 (mAb 5OH.19-Alexa 488) were generated using Alexa 488 and Alexa 594 labelling kits purchased from Molecular Probes (Eugene,Or.). Fab fragments and mAbs were suspended individually in PBS at a final concentration of 2 mg/mL at room temperature. Antibody preparations were then combined with the contents of one vial of unreacted Alexa 594 or Alexa 488

fluorescent dye for 1 hour. Following conjugation unreacted dye was removed by passage through a size exclusion column with a molecular cut-off of 15 000 MWT. Retention of binding specificity was determined by competitive labelling using unconjugated mAb 7H.3. Here 1 x 10^5 Raji cells were pre-treated in the presence or absence of mAb 7H.3 at 10 mg/mL in 100 µL of PBS at 4^0C. Following pretreatment with unlabelled MHC class II cells were treated with 5 mg/mL of directly conjugated 7H.3 or 7H.3 Fab fragments for an additional 45 min in 100 µL of PBS on ice. Cells were then washed in two 1 mL volumes of PBS and analysed by flow cytometry for the ability of unlabelled mAb 7H.3 to block labelling. For anti-CD9 direct conjugates (50H.19-Alexa 488) competitive binding assays were again carried out using unlabelled mAb 50H.19.

Fab fragments were generated using a kit purchased from Pierce chemicals. In brief 5 mg of mAb 7H.3 were incubated with immobilized Papain for 4 hours at 37^0C in a shaker water bath. Uncleaved mAb 7H.3 and Fc fragments were removed by binding to a Protein A Sepharose column and Fab fragments were collected from the column eluate. Fab fragments were further purified by dialysis in PBS for 24 hours with three changes of the dialysis buffer followed by two additional volumes of distilled water. Fab fragments were then lyophilized and resuspended to 2 mg/mL in PBS.

Section 2.2. Cell Culture

Raji B-lymphoma cells are grown in Gibco culture media RPMI 1640, pH 7.5, supplemented with 10% fetal calf serum (FCS) (RPMI/FCS). Cells are maintained at low cell density (1.0-3.0x10^5 cells /mL). Raji/CD9 transfectant cells were generated by transfection of a pCDNA3.1$^-$ plasmid encoding a single copy of the human CD9 cDNA transcript. Stably transfected CD9$^+$ cells are selected by FACS for surface expression using the directly conjugated monoclonal anti-CD9 antibody mAb 50H.19-FITC. Drug pretreatments for individual experiments are described in detail elsewhere. In brief, MBCD treatments (5-10 mM) were carried after harvesting and resuspending cells in RPMI 1640, pH 7.5, supplemented with 0.1% BSA (RPMI/BSA) at 37^{0}C in a 5% CO_2 incubator. To inhibit actin polymerization, cells were pretreated with cytochalasin D (1μM) or latrunculin A (2 μM) for 60-90 min in RPMI/FCS at 37^{0}C in a 5% CO_2 incubator.

Section 2.3. Immunoprecipitation

Co-immunoprecipitation of CD9 by anti-MHC Class II mAb 7H.3 was investigated from 2x10^6 Raji/CD9 transfectant cells lysed in 1% CHAPS. Cells were harvested and resuspended in 100 μL of RPMI/FCS on ice with 10 μg/mL mAb 7H.3 for 45 min. Unbound mAb 7H.3 was removed by three consecutive washes in PBS, pH 7.5 leaving only plasma membrane MHC class II bound mAb 7H.3. Cells were then extracted into 500 μL of 1% CHAPS lysis buffer (50 mM Tris pH 7.4, 137 mM NaCl, 1% w/v

CHAPS, containing leupeptin 2.0 μg/mL, aprotinin 2.0 μg/mL, pepstatin 2.0 μg/mL, antipain 2.0 μg/mL, iodoacetamide 1.0 mM) for 90 min at 4^0C in a rotating mixer. Following detergent extraction a cleared lysate was generated by centrifugation of the cell lysate at 14 000 rpm for 10 min in a benchtop centrifuge followed by removal of the insoluble pellet. Antibody ligated surface MHC class II was recovered by addition of 30 μL (1:1, beads:PBS) of Protein A Sepharose beads to the cleared lysate for 90 min at 4^0C in 1% CHAPS lysis buffer. Protein A bound immuno-precipitates were isolated by a brief 30 sec centrifugation at 14 000 rpm in a benchtop centrifuge. Protein A Sepharose beads were then washed in an additional two 1 mL volumes of 1% CHAPS lysis buffer. The beads are the isolated by a brief centrifugation at 14 000 rpm and bound protein complexes solublized in 40 μL of 1 X SDS running buffer. Samples were loaded onto a 5-20% continuous-gradient polyacrylamide gel and separated by application of a 200V current for 50 min in a BioRad mini-PROTEAN II cell electrophoresis apparatus. Separated proteins were transferred overnight (30V) onto nitrocellulose and blotted with directly conjugated anti-CD9-HRP (50H.19-HRP) or polyclonal rabbit anti-MHC class II followed by anti-rabbit-HRP. CD9 and MHC class II are detected by enzyme-linked chromatography (ECL) using substrates obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK.).

Section 2.4. Analysis of The Detergent Insoluble Fraction

2.4.1. Buoyant Density Centrifugation of CD9 from a 1% CHAPS Whole Cell Lysate.

$2x10^6$ Raji/CD9 transfectant cells were incubated in 500 µL RPMI/FCS, pH 7.4, in the presence or absence of 10 µg/mL mAb 50H.19 for 60 min at 4^0C, Cells were then washed in two 1.0 mL volumes of the RPMI/FCS and resuspended in RPMI/FCS with or without exposure to 10 µg/mL rabbit anti-mouse polyclonal Ab and incubated at 37^0C for an additional 60 min. Following incubation cells were washed in two 1.0 mL volumes of PBS, pH7.5 followed by resuspension in 200 µL of 1% w/v CHAPS lysis buffer supplemented with 10% sucrose and incubated in a rotating mixer for 90 min at 4^0C in order to generate a whole cell lysate.

Following detergent extraction, 400 µL of Optiprep density gradient preparation is added to the 200 µL of lysate generating a final sucrose concentration of 40%. The 600 µL sample was then transferred to the bottom of a 5 mL centrifuge tube and a 35-10% discontinuous gradient was layered overtop the 40%, 0.6 mL sample. The final 40%-10% discontinuous gradient was prepared by overlaying the 40% sucrose sample with 0.6 mL volumes of 1% CHAPS containing Optiprep/1% CHAPS lysis buffer gradient preparations at 35%, 30%, 25%, 20%, and 10% sucrose. Samples were then centrifuged at 165 000g for 4 hours in an ultracentrifuge.

After buoyant density centrifugation, samples were fractionated into six 0.6 mL volumes. Proteins from each fraction were precipitated by addition of 100% w/v TCA to a final concentration of 10% w/v. Precipitated sample proteins were recovered by centrifugation at 14 000 rpm for 10 min in a benchtop centrifuge. Recovered proteins were then resolubilized through addition of 20 µL 1.0 M Tris buffer, pH 8.0, and 20 µL of 2x SDS non-reducing running buffer. Samples were then loaded onto a 5-20% continuous-gradient polyacrylamide gel for separation. Samples proteins were then separated by application of a 200V current for 50 min in a BioRad mini-PROTEAN apparatus. Separated proteins were then transferred overnight (30V) onto nitrocellulose and blotted with 50H.19-HRP conjugated antibodies. Labelled protein was detected by ECL using reagents purchased from Amersham Pharmacia.

2.4.2. Buoyant Density Centrifugation of MHC class II from a 1% CHAPS Whole Cell Lysate.

$2x10^6$ Raji/CD9 transfectant cells were incubated in 500 µL RPMI/FCS, pH 7.4, in the presence or absence of 10 µg/mL mAb 7H.3 or isotype matched mAb UPC10 for 60 min at 4^0C. Cells were then washed in two 1.0 mL volumes of the RPMI/FCS and resuspended in RPMI/FCS with or without exposure to 10 µg/mL rabbit anti-mouse polyclonal Ab. Cells were then treated as described for buoyant density centrifugation and electrophoresis of CD9. Nitrocellulose blots are indirectly analysed for the distribution of mAb bound MHC class II by blotting for the presence of mouse IgG

using goat anti-mouse-HPR polyclonal Ab as a blotting antibody for MHC class II was not yet available.

2.4.3. Analysis of MHC class II within the detergent insoluble fraction of a post-nuclear fraction by buoyant density centrifugation.

Samples were obtained from $5x10^6$ Raji/CD9 transfectant cells. Here cells were preincubated in 500 µL of culture supernatant at 4^0C for 60 min in the presence or absence of 10 µg/mL mAb 7H.3. Cells were then washed in two 1.0 mL volumes of RPMI/FCS followed by resuspension in 500 µL of the same in the presence or absence of 10 µg/mL of polyclonal rabbit anti-mouse crosslinking polyclonal Ab and incubated for 20 min at 37^0C.

Following exposure to secondary antibodies cells were rapid-cooled by exposure to a -70^0C acetone/dry ice bath to minimize disruption of raft components during cooling stages. Cells were then resuspended and washed in two 1.0 mL volumes of ice cold cytoskeletal stabilizing buffer (CSB) (PIPES 10 mM, KCl 100 mM, MgCl 2.5 mM, CaCl 1.0 mM, pH 7.5) described previously by Geppert and Lipsky [57] containing protease inhibitors (leupeptin 2.0 µg/mL, aprotinin 2.0 µg/mL, pepstatin 2.0 µg/mL, antipain 2.0 µg/mL, iodoacetamide 1.0 mM) to remove unbound Ab and media components. Cell suspensions were then lysed by Dounce homogenisation to release cytoplasmic and nuclear components. Homogenised cells were then centrifuged at 3000 rpm for 5 min to pellet nuclei and intact cells. The supernatant was then

subjected to a high-speed (14 000 rpm) spin for 10 min to isolate the post nuclear fraction.

The pellet containing the post nuclear fraction was resuspended in 200 µL of CSB supplemented with 1% w/v CHAPS, 10% sucrose and protease inhibitors and incubated for 90 min at 4^0C. Following detergent extraction 400 µL of Optiprep density gradient was added to a final sucrose concentration of 40% and samples were prepared for buoyant density centrifugation and electrophoresis as described in section 4.2.1. MHC class II bound to nitrocellulose was blotted with polyclonal rabbit anti-MHC class II followed by goat anti-rabbit-HRP. Antibody labelled MHC class II is detected by ECL as described previously.

Section 2.5 Confocal microscopy

2.5.1. Fixed cell Confocal Microscopy

$1x10^6$ Raji/CD9 cells were harvested washed in two 1.0 mL volumes of ice cold PBS then resuspended into 1.0 mL of PBS containing 2% PFA on ice for 10 min. Following fixation, the cells were resuspended in PBS + 0.1% BSA (PBS/BSA) containing 10 µg/mL each of anti-CD9 mAb 50H.19-Alexa 488 and anti-MHC class II mAb 7H.3-Alexa 594 on ice for an additional 45 min. After staining cells were washed in two 1.0 mL volumes of ice cold PBS/BSA then placed in 2.0 mL of PBS/BSA in 35 mm coverglass-bottom petri dishes for confocal microscopy. Images were taken on a Zeiss

Axiovert 510 laser scanning microscope using HeNe and Argon lasers emitting at 594 nm and 488 nm respectively. 2-Dimentional images are shown through the mid section of the cell.

2.5.2. Live Cell Confocal Microscopy

Cells for live imaging by confocal microscopy were harvested ($5x10^5$ cells) from a culture of Raji/CD9 transfectant cells and resuspended in 0.1 mL of RPMI/FCS on ice. Cells were incubated with 10 μg/mL anti-MHC Class II mAb 7H.3-Alexa 594 and anti-CD9 mAb 5OH.19-Alexa 488 for 45 min on ice. Excess antibody was removed by two serial washes in 1.0 mL volumes of ice-cold culture supernatant before resuspension in coverglass-bottom 35 mm petri dishes in 2.0 mL of 37^0C supernatant. Cultures were over-laid with 1.0 mL of embryo-tested mineral oil (Sigma; Oakville, On), to minimize evaporation, and mounted in an incubation chamber at 37^0C flushed with 5% CO_2 on the microscope stage. Confocal images were taken with a Zeiss 510 Axiovert laser scanning confocal microscope using Argon and HeNe lasers exciting at 488 nm and 543 nm respectively. Reconstructed 3-dimensional images are generated from a Z-stack taken at an interval of 0.25 μm using Zeiss LSM 510 software.

2.5.3. Detergent extraction for Confocal Microscopy

Triton X-100 detergent extracted cells were prepared from $5x10^5$ cells isolated from a subconfluent culture ($1.0\text{-}3.0x10^5$ cells /mL). Cells were resuspended in 0.1 mL of

culture supernatant on ice and treated with anti-MHC Class II mAb 7H.3-Alexa 594 or anti-β1 integrin mAb AP1.138 for 60 min. Where appropriate crosslinking with rabbit anti-mouse polyclonal Ab was carried out in 0.1 mL of culture supernatant at 37^{0}C for 60 min. Cells were then washed with two 1.0 mL volumes of ice-cold culture supernatant and resuspended in 0.1 mL of CSB/BSA and loaded onto poly-L-lysine coated coverglass-bottom petri dishes at 37^{0}C for 10 min. Cells were then washed in two 2.0 mL volumes of ice-cold CSB/BSA buffer. To remove detergent soluble surface labelled MHC class II detergent extraction was carried out in 35 mm petri dishes by overlaying cells with CSB containing 0.5% Triton X-100 and protease inhibitors for 5 min with gentle agitation. Triton X-100 was removed by an additional wash in ice-cold CSB +0.1% BSA (CSB/BSA). Extracted cells were fixed in CSB for 10 min at 4^{0}C by addition of paraformaldehyde (PFA) to a final concentration of 2% *in situ*. F-actin staining was preformed at 4^{0}C after fixation by addition of phalloidin-FITC (1.0 μg/mL) for 60 min. The final cell preparation was viewed under the Zeiss 510 Axiovert microscope using Argon and HeNe lasers exciting at 488 and 543 nm respectively.

Colocalization of MHC Class II and CD9 within the 1% CHAPS detergent insoluble fraction was determined from 5x10^5 cells stained with 10 μg/mL anti-MHC class II mAb 7H.3-Alexa 594 and anti-CD9 mAb 5OH.19-Alexa 488 for 60 min on ice. Following antibody labelling cells were washed in three 1.0 mL volumes of CSB/BSA before resuspension in CSB + 1% CHAPS under gentle agitation for 5 min at 4^{0}C. Cells were then washed in two 1.0 mL volumes of CSB/BSA then fixed in CSB + 2%

PFA for 10 min at 4^0C. Cells were recovered and resuspended in 2.0 mL of CSB/BSA and placed in the bottom of a coverglass-bottom petri dish for confocal microscopy. Triton X-100 extracted cells were treated as described for 1% CHAPS with the exception of 0.5% v/v Triton X-100 substitution for 1.0% w/v CHAPS in the CSB extraction buffer.

2.5.4. 4-Dimensional Confocal Microscopy: CD9 and MHC class II Internalization

Internalization of mAb 7H.3-Alexa 594 ligated MHC Class II was followed in live cells mounted on a 37^0C, CO_2 flushed incubation chamber located on the Zeiss Axiovert 510 microscope stage. Surface MHC class II was labelled with 10 µg/mL mAb 7H.3-Alexa 594 in 100 µL of culture supernatant on ice to prevent internalization during staining. Cells were then washed in two 1.0 mL volumes of ice cold culture supernatant to remove unbound mAb 7H.3-Alexa 594. Initiation of internalization (time zero) was marked by resuspension of cells in 2.0 mL of 37^0C culture supernatant in 35 mm coverglass-bottom petri dishes coated with poly-L-lysine. Label internalization was followed by 4-dimensional confocal microscopy. In brief, 3-dimensional images were generated from Z-stacks produced under a 63x lens using a 1.0x field of view and a depth interval of 0.50 µm at specified time points. Analysis of internalized label was accomplished using Bitplane Imaris 3-dimensional imaging software. Integration of voxels (the 3-dimensional representation of a 2-dimensional pixel) within user-defined regions of each individual cell within the 3-dimensional image was used for quantification. Internalization of labelled MHC class II was

quantified by calculating the proportion of total cell label (surface + cytoplasmic) to the proportion of cytoplasmic label alone. The proportion of internalized material was described as a percent of the total label at a specified time point following initiating of internalization and represents the average of all cells imaged within a given experiment. Pretreatment of cells with latrunculin A, and cytochalasin D was carried out in RPMI/FCS in a 37^0C, 5% CO_2 flushed incubator for 60 min prior to staining. MBCD pretreatments were carried out under identical conditions for 30 min in RPMI/BSA. Prior to staining, cells are placed on ice for 10 min to inhibit internalization prior to addition of directly conjugated 7H.3-Alexa 594. During 4-dimentional confocal microscopy cells are maintained in RPMI/FCS (latrunculin A and cytochalasin D) or RPMI/BSA (MBCD) containing appropriate inhibitors.

Section 2.6. Flow cytometry

2.6.1. Triton X-100 Detergent Extraction: Cytoskeletal association

Analysis of MHC Class II and CD9 retention within the Triton X-100 detergent insoluble fraction was analyzed from $1x10^6$ cells isolated from a subconfluent cell culture. Cells were treated in RPMI/FCS containing increasing concentrations of latrunculin A for 60 min at 37^0C. Cells were then harvested and resuspended in 0.2 mL of the same RPMI/FCS containing latrunculin A in the presence of 10 µg/mL anti-MHC Class II mAb 7H.3-Alexa 488 or anti-CD9 mAb 50H.19-Alexa 488 for 1 hour. After staining unbound antibody was removed by two serial washes in 1.0 mL

volumes of CSB + 0.1% BSA containing protease inhibitors (leupeptin 2.0 μg/mL, aprotinin 2.0 μg/mL, pepstatin 2.0 μg/mL, antipain 2.0 μg/mL, and iodoacetamide 1.0 mM). Cells are extracted in CSB containing 0.5% Triton X-100 + 0.1% BSA and protease inhibitors for 5 min on ice to remove detergent soluble MHC class II from the cell surface. Cells were then washed in two additional 1.0 mL volumes of CSB/BSA and resuspended in 1.0mL of the same for analysis by flow cytometry using a Becton Dickinson FACSort.

2.6.2. Transferrin and BSA Internalization: flow cytometry

Transferrin uptake was monitored after addition of transferrin-FITC (50 μg/mL) to $1x10^6$ cells suspended in 1.0 mL of RPMI/BSA at 37^0C. Cells were maintained throughout the experiment at 37^0C in a water bath. Following addition of transferrin-FITC at time zero, 0.1 mL fractions were removed at specified time points and placed on ice with addition of NaN_3 to a final concentration of 0.1% v/v. NaN_3 is added to prevent further internalization or fusion of endocytic vesicles with the plasma membrane prior to analysis. Sample fractions were washed in two volumes of ice cold RPMI/BSA, resuspended in 0.5 mL of RPMI/BSA and then analysed by flow cytometry. The level of BSA internalization was quantified as a measure of relative fluorescence to control cells that were incubated in the presence of 0.2% BSA-FITC and 0.1% v/v NaN_3 on ice for each time point analyzed. Pretreatment of cells with 5 mM MBCD was carried out in RPMI/BSA for 30 min in a 37^0C, 5% CO_2 flushed

incubator. Internalization of MBCD cholesterol treated cells was carried out in RPMI/BSA under identical conditions to those outlined above.

BSA internalization was monitored following resuspension of $5x10^6$ cells in 5.0 mL of RPMI/BSA. Cells were placed in a 37^0C water bath after FITC conjugated BSA was added to a final concentration of 0.2% w/v at time zero. 0.1 mL fractions were removed and placed on ice in 0.1% NaN_3 and prepared for flow cytometry analysis of mean fluorescence as described above. Pretreatment of cells with MBCD is carried out as described in section 2.5.4.

Chapter 3: Results. CD9 Regulation of MHC class II association with the Detergent Insoluble Fraction on the Surface of B-Lymphocytes

Section 3.1. Chapter Introduction and Experimental Objectives

Both the actin-based cytoskeleton and the lipid raft components of the detergent insoluble membrane may be implicated in normal MHC class II restricted antigen presentation. Interaction of MHC class II association with either component of the detergent insoluble fraction could have a profound impact on both classical and non-classical function. An essential prerequisite for MHC class II signalling function involves receptor clustering. T-cell surveillance of antigen presenting cells requires engagement of adhesion receptor pairs, co-receptors and TCR-MHC class II-peptide complexes for early peptide recognition. Following initial foreign peptide recognition, productive T-cell activation events demand the formation of approximately 200 productive TCR-MHC class II-peptide interactions [100]. Furthermore, multivalent clustered MHC-class II-peptide complexes appear to be more efficient at stimulating T-cell responses to foreign epitopes [65]. Association of MHC class II with either the cortical actin cytoskeleton or the lipid raft component of the plasma membrane may therefore provide the conditions necessary for effective T-cell activation *in vivo*.

Anchoring MHC class II to the actin cytoskeleton could retain MHC class II within regions of active signalling such as the immunological synapse described by Dustin *et*

al. [67]. By limiting lateral diffusion out of a region of active TCR-MHC class II-peptide engagement, the actin cytoskeleton increases the effective valency of MHC class II-peptide epitopes within a defined cell-cell contact zone. Multivalent interaction of T-cell and B-cell cognitive proteins in a concentrated region may shift the local balance of signals towards activation. Association of MHC class II with the cortical actin cytoskeleton could further concentrate epitopes within the T-cell/B-cell contact zone by directing lateral MHC class II trafficking within the plasma membrane. Wulfing *et al.* have observed that beads attached to T-cell adhesion receptors translocate to regions of TCR engagement in a process which depends on myosin motor proteins and association of the major co-stimulatory receptor pair, B7-CD28 [69]. Interaction of T-cell adhesion receptors with the cortical actin cytoskeleton may potentiate both "inside-out" and "outside-in" signalling events. LFA-1 provides "outside-in" co-stimulatory signals in the region of TCR engagement promoting productive activation events [101]. In parallel, "inside-out" signals are thought invoke conformational changes in adhesion receptors resulting in high affinity ligand binding with an opposing cell membrane and promote actin accumulation under the region of TCR engagement [101] that stabilizes the T-cell/B-cell synapse. On the opposing B-cell membrane association of MHC class II molecules with the cortical actin cytoskeleton may act to corral cognitive receptors, downstream signalling effectors, co-stimulatory and adhesion molecules into a similar array necessary for B-cell activation.

As with cytoskeletal anchorage entrance of MHC class II into the lipid raft component of the plasma membrane could result in lateral segregation of MHC class II on the surface of antigen presenting cells. Crosslinking MHC class II molecules on the surface of THP-1 T-lymphocytes induces synergistic co-aggregation with FITC-cholera toxin which binds GM1, a marker of membrane rafts [75], and accumulation within the low buoyant density fraction of the Triton X-100 detergent insoluble membrane. Studies into the regulation of cell surface receptors have revealed a subset of proteins capable of entering lipid raft following antibody crosslinking [57]. Entrance of cross-linked receptors into the lipid raft is thought to parallel fusion of raft microdomains into larger subdomains a process that can be directly visualized through fluorescence microscopy.

Clustering of small lipid raft domains on the plasma membrane of B-cells may precede formation of large signalling regions. Such regions may contain distinct subspecies of surface receptors and downstream effector molecules generating a cognitive signalling complex with highly regulated constituent proteins. Lateral segregation of cognitive molecules represents a model for co-ordination of lymphocyte activation. By this model, signal integration at the plasma membrane would generate a single coordinated "voice" for communication to the nucleus through conserved signalling pathways. In support of a model involving lateral segregation, members of the Src tyrosine kinase family have also been identified as constituents of lipid rafts [102]. MHC class II having a relatively short cytoplasmic tail is thought to require protein-protein interaction to generate activation signals following TCR engagement. Access of MHC

class II to preformed signalling complexes within the signal-enriched pools present within lipid rafts may reduce the requirement for MHC class II to independently assemble multimeric protein complexes. Members of the tetraspanin family present within lipid rafts are known to associate with MHC class II. Among these are CD9 and CD81 [80, 103]. Described as molecular facilitators, the tetraspanins associate with a number of signalling complexes including those involving β-integrins [94, 95, 104-108] and the co-stimulatory molecule CD19 [86] and could provide the information required for cognitive complex formation. As such resident signalling complexes within lipid rafts need only be accessed via entrance into the highly ordered lipid environment for successful initiation of activating signals. As a result, dynamic regulation of access to an environment suitable for signalling may alter the potential outcome of MHC class II non-classical function.

Little is known about the regulation of MHC class II lateral sorting and clustering following TCR engagement at the APC surface. Identification of mechanisms directing MHC class II into the T-cell/B-cell interface and regulation of access to downstream effector molecules are essential to understanding the consequences of T-cell/B-cell activation following antigen presentation. Studies into the field of MHC class II signalling suggest that downstream signalling by MHC class II following antibody crosslinking results in association with the cortical actin cytoskeleton. However, these studies were based solely on the observation that MHC class II is enriched following crosslinking within the detergent insoluble fraction and failed to distinguish between the high buoyant density actin enriched component and the low buoyant density lipid

raft components [52, 109] of the insoluble fraction. Recently, Huby *et al.* [75] have implicated MHC class II as an inducible resident of lipid rafts. In these studies antibody induced crosslinking of MHC class II in THP-1 cells translocated a portion of MHC class II into the triton X-100 low buoyant density fraction and this redistribution into lipid rafts was upstream of phosphotyrosine signalling. *Here we provide evidence that MHC class II is associated with the actin-based cytoskeleton in a manner that is dependent upon the integrity of lipid rafts. Furthermore, we provide evidence that association of MHC class II with CD9, a member of the tetraspanin superfamily and resident of lipid rafts, modulates the ability of MHC class II to form associations with the cortical actin cytoskeleton through a raft-dependent process.*

Section: 3.2. Association of MHC class II and CD9 on the surface of Raji/CD9 transfectant mature B-cells

3.2.1. Are MHC class II and CD9 associated on the surface of the mature transfected B-cell line Raji/CD9?

We and others [56], have demonstrated that MHC class II and CD9 are capable of forming associations when co-expressed on the plasma membrane. In order to demonstrate the plasma membrane distribution of CD9 and MHC class II on Raji/CD9 transfectant cells, directly fluorescent conjugated anti-MHC class II monoclonal antibodies were used to stain fixed cells. $5x10^5$ cells were treated with 2% PFA followed by labelling with directly conjugated anti-CD9 (50H.19-Alexa 488) and anti-

MHC class II (7H.3-Alexa 594). Using a laser scanning confocal microscope, direct immunofluorescence was used analyze the plasma membrane distribution of CD9 and MHC class II. A cross sectional image taken through the midsection of a cell reveals colocalization of MHC class II and CD9 on the surface of transfectant Raji/CD9 B-cells (Figure 1). Furthermore MHC class II and CD9 are both present within highly convoluted membrane extensions of the plasma membrane.

Next we asked whether simultaneous ligation of live cells with mAb 50H.19-Alexa 488 and mAb 7H.3-Alexa 594 could disrupt colocalization of CD9 with MHC class II. In order to visualize the surface distribution of CD9 and MHC class II in Raji/CD9 transfectant cells following ligation with directly conjugated anti-MHC class II mAb 7H.3-Alexa 594 and anti-CD9 mAb 50H.19-Alexa 488, we employed 3-dimensional confocal microscopy imaging. Live cell 3-dimensional imaging of Raji/CD9 transfectant cells 20 min post-ligation revealed colocalization of MHC class II and CD9 on the plasma membrane. Ligation of CD9 did not therefore appear to dissociate MHC class II from CD9 (figure 2). During the experiment a proportion of antibody-tagged MHC class II translocated to an internal compartment that probably corresponds to the peptide-loading compartment (white arrows). Similar 3-dimentional images taken with a lower magnification of cells fixed prior to warming and following incubation for 25 min at 37^0C reveals that a proportion of MHC class II bound 7H.3 enters the cell following ligation independent of staining and fixation conditions (Figure 2, bottom panel). To investigate the physical association of MHC class II and CD9 on the plasma membrane we asked whether anti-MHC class II mAb 7H.3

Figure 1. *Colocalization of MHC Class II with CD9 on the surface of Raji/CD9 transfectant cells.* Cells are fixed with paraformaldehyde then stained with anti-CD9 mAb 5OH.19-Alexa 488 and anti-MHC Class II mAb 7H.3-Alexa 594. Images are representative of cross sections taken through the midsection of the cell. Both MHC class II (A) and CD9 (B) are present within filopodia and colocalize within regions of intense staining on the plasma membrane. No internal pools of MHC class II or CD9 are present indicating that the mAb do not gain access to an internal compartment prior to incubation at 37°C.

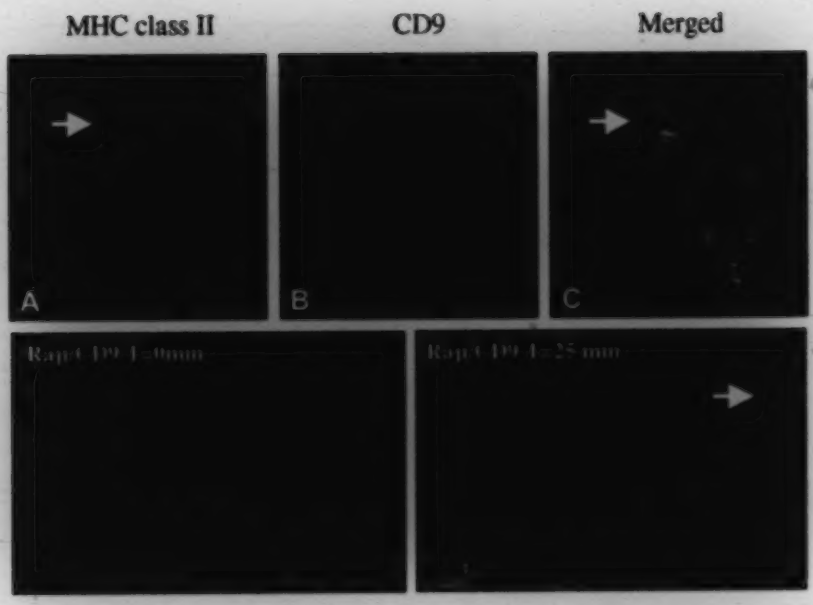

Figure 2. *Co-localization of MHC class II with CD9 on the surface of live Raji/CD9 transfectant cells.* Raji/CD9 cells were stained with (A) anti-MHC Class II mAb 7H.3-Alexa 594 or (B) anti-CD9 mAb 50H.19-Alexa 488. Three dimensional images were generated from a series of Z-stacks located at 0.2 μm intervals through the central 1/3rd of the cell. On the surface of live cells MHC class II (A) and CD9 (B) are colocalized. Internalized anti-MHC class II mAb 7H.3 Alexa 594 is observed following incubation at 37°C (white arrows) but is absent in cells fixed prior to warming (T=0min).

directed at surface MHC class II molecules could co-immunoprecipitate CD9. Raji/CD9 cells were treated on ice for 60 min with mAb 7H.3 to label the surface MHC class II pool. Cells are neither permeablized nor capable of internalizing bound 7H.3 under these conditions of staining since mAb cannot access to the internal pool of MHC class II as is indicated by the lack of internal 7H.3-Alexa 594 label at T=0min (figure 2). Blotting MHC class II and CD9 from a 1% CHAPS detergent lysates revealed that mAb 7H.3 associates with an amount of CD9 equivalent to that obtained by direct immunoprecipitation with the anti-CD9 mAb 50H.19 (Figure 3). This indicates that the two proteins are associated at a high stoichiometric ratio. Interestingly, the anti-CD9 mAb did not reciprocally co-immunoprecipitate MHC class II. Ligation of CD9 with mAb 50H.19 may therefore interfere with the association of MHC class II and CD9, or alternatively the epitope for the anti-CD9 mAb is masked in CD9/MHC class II complexes. Consequently, it seems likely that colocalization of CD9 with MHC class II observed by confocal microscopy indicates that CD9 colocalizes with MHC class II associated CD9. This would be compatible with the notion that CD9 can self-associate into channel-like structures. Alternatively CD9 can access similar regions of the plasma membrane independent of MHC class II.

3.2.2. Are MHC class II and CD9 residents of the same lipid raft component of the detergent insoluble fraction?

Previously we have observed that both MHC class II and CD9 are present within the low buoyant density component of the 1% CHAPS detergent insoluble fraction when

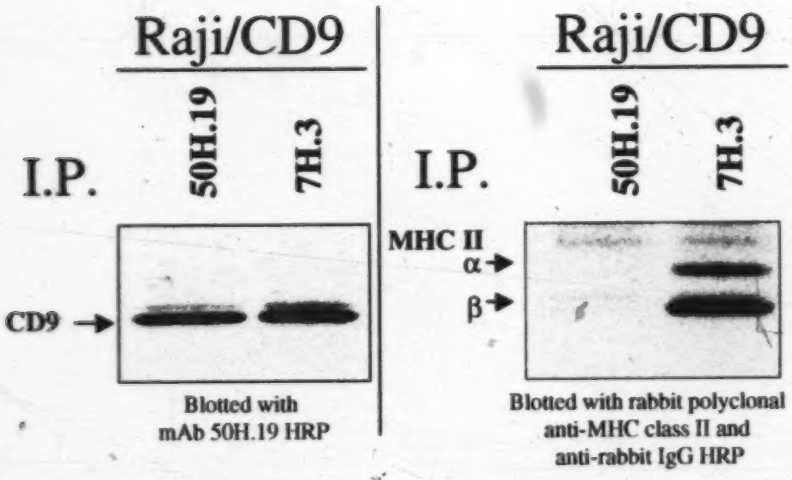

Figure 3. *Co-immunoprecipitation of CD9 with anti-MHC class II mAb 7H.3 from the surface of Raji/CD9 transfectant cells in CHAPS detergent.* Raji/CD9 transfectant cells were treated with anti-MHC class II mAb 7H.3 on ice to label surface MHC class II.. Cells were then lysed in 1% CHAPS. Samples were immunoprecipitated and analyzed by 5-20% non-reducing PAGE. Anti-MHC class II mAb 7H.3 co-immunoprecipitates CD9 from the 1% CHAPS lysate (A), however, anti-CD9 mAb 50H.19 was unable to co-immunoprecipitate MHC class II under the same conditions (B).

analysing whole cell lysates by buoyant density centrifugation. However, coincidence within the low density fraction does not necessarily imply that both CD9 and MHC class II are located in the same lipid rafts. We have shown that CD9 and MHC class II associate at the plasma membrane by co-immunoprecipitation from 1% CHAPS detergent lysates (Figure 3). If MHC class II and CD9 interact in the lipid raft environment then both proteins should colocalize within the same detergent insoluble region of the plasma membrane. We therefore investigated whether CD9 and MHC class II colocalize on the surface of detergent extracted cells. Raji/CD9 transfectant cells were examined by confocal microscopy following the detergent extraction technique described by Geppert and Lipsky [57] for the study of cytoskeletal associated proteins in T-lymphocytes. In brief, following labelling of surface MHC class II and CD9 with directly fluorescent conjugated anti-CD9 and anti-MHC class II, cells were extracted into CSB supplemented with either 1% CHAPS or 0.5% Triton X-100 for 5 min. Extracted cells were fixed and examined under the laser scanning confocal microscope. Examination of the 1% CHAPS detergent extracted cells reveals a high level of retention of both MHC class II and CD9 on the surface of Raji/CD9 transfectant cells (Figure 4; Lanes A-C). Furthermore MHC class II and CD9 colocalize within enriched domains often associated with areas of membrane ruffling and filopodia. The high level of label retention observed is a function of mild detergent extraction. When cells were extracted by 0.5% Triton X-100, regions of concentrated label retention were observed for both anti-MHC class II and anti-CD9 bound surface receptors (Figure 4; C-F) although the levels of retention were less than for cells extracted into 1% CHAPS as might be expected for detergents that maintain protein-

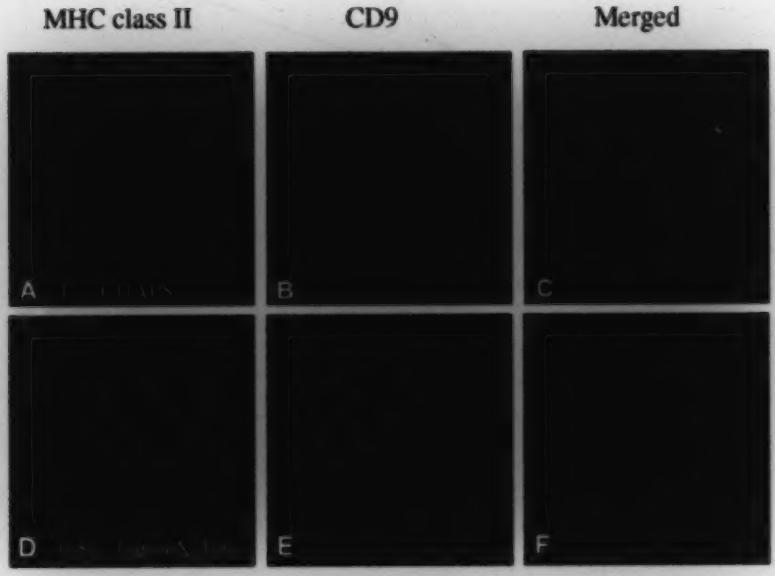

Figure 4. *Colocalization of MHC Class II with CD9 in the CHAPS and Triton X-100 detergent insoluble fraction.* Raji/CD9 transfectant cells were treated with with anti-MHC Class II mAb 7H.3-Alexa 594 (A and D) and anti-CD9 mAb 5OH.19-Alexa 488 (B and E). Following antibody labeling cells were extracted into 1% CHAPS or 0.5% Triton X-100, fixed, and viewed under the confocal scanning microscope. 0.5% Triton X-100 detergent extracted cells show distinct patches of insoluble material at the cell periphery whereas 1% CHAPS extracted cells show a more continuous distribution of CD9 and MHC class II retention on the cell surface. Furthermore, MHC class II and CD9 colocalize to the same regions of detergent insolubility in both 1% CHAPS and 0.5% Triton X-100. Merged images (C and F). MHC class II and CD9 are retained at the surface of both 1% CHAPS and 0.5% Triton X-100 extracted cells. 1% CHAPS detergent extracted cells retain enhanced levels of MHC class II and CD9 at the cell surface.

protein interactions. Colocalization of MHC class II and CD9 within 1% CHAPS and 0.5% Triton X-100 detergent insoluble regions of the plasma membrane indicate that they likely participate within the same lipid rafts.

Section 3.3. Dynamic translocation of MHC class II and CD9 into the lipid raft component of the detergent insoluble fraction

3.3.1. What is the distribution of CD9 and MHC class II between the soluble and insoluble membrane?

Since MHC class II and CD9 appear to be residents of the same detergent insoluble fraction in Raji/CD9 transfectants, we looked at the distribution of CD9 and MHC class II by evaluation of buoyant density gradients. Previously, Triton X-100 detergent has been employed for isolation of lipid raft components of the plasma membrane. Here, we have employed the zwitterionic detergent CHAPS in the preparation of detergent insoluble fractions. 1% CHAPS detergent lysates have been used to investigate protein-protein interactions by co-immunoprecipitation. It is thought that 1% CHAPS is able to maintain weaker non-covalent protein-protein interactions. In order to evaluate the dynamics of CD9 distribution on buoyant density gradients we examined the effect of antibody ligation and crosslinking on the CHAPS detergent insoluble fraction. Raji/CD9 cells were treated with or without mAb 50H.19 at 4^0C for 60 min. Following ligation of primary antibody cells were treated in the presence or absence of rabbit anti-mouse polyclonal antibody to induce crosslinking in RPMI/FCS

for 60 min at 37^{0}C. Following antibody treatment cells were lysed in a 1% CHAPS buffer. The detergent insoluble material was recovered by centrifugation and applied to a 10-40% discontinuous sucrose gradient for ultra-centrifugation. At resting state (untreated cells) CD9 is distributed between both the low (insoluble) and high-density (soluble) component of 1% CHAPS detergent lysed cells. The majority of protein appears to reside within the higher density region of the gradient (Figure 5; lane A). Pretreatment of Raji/CD9 transfectant cells with anti-CD9 mAb 50H.19 promoted translocation of a small but significant portion of CD9 from high to low density fractions (Figure 5; lane C). By contrast treatment of cells with rabbit anti-mouse mAb had no significant effect on the distribution of CD9. Crosslinking antibody-labelled CD9 with rabbit anti-mouse polyclonal antibodies further translocated CD9 into the low buoyant density component (Figure 5; lane D). Previous experiments in or lab have demonstrated that the two low buoyant density fractions (20% and 10% sucrose) are sensitive to treatment with MBCD (Andrew Shaw, personal communication) indicating that these fractions likely represent lipid raft associated proteins. Partitioning of CD9 into the low buoyant density fraction suggests that one consequence of clustering CD9 is to influence its immediate lipid environment.

Next we asked whether MHC class II distribution could be altered under the same conditions of antibody ligation in Raji/CD9 transfectant cells. A similar experiment carried out on MHC class II points to subtle differences in the ability of MHC class II and CD9 to translocate into lipid rafts. Unlike CD9, engagement of MHC class II with mAb alone is unable to induce translocation of MHC class II into the 1% CHAPS

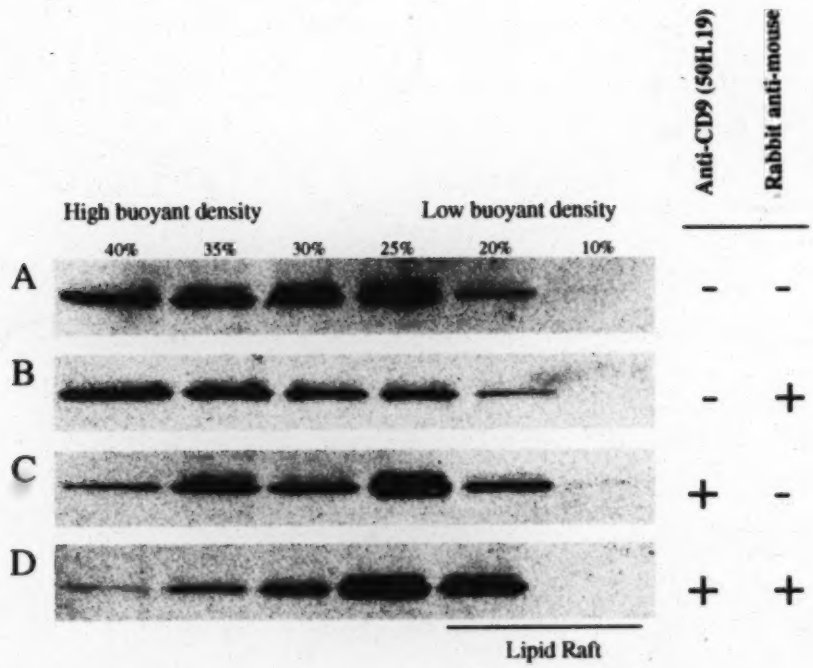

Figure 5. *Antibody induced clustering of CD9 alters its buoyant density distribution within 1% CHAPS detergent lysates.* Raji/CD9 transfectant cells were treated with or without anti-CD9 mAb 50H.19 for 60min at 4°C, then incubated with or without rabbit anti-mouse heavy and light chain polyclonal antibody for 60 min at 37°C. Cells were then lysed in 1% CHAPS buffer and subjected to buoyant density centrifugation followed by separation using 5-20% non-reducing PAGE conditions. Samples were transferred to nitrocellulose, blotted with 50H.19-HRP mAb and resolved by ECL. Treatment of Raji/CD9 cells with anti-CD9 alone or followed by cross-linking secondary antibodies is sufficient to translocate CD9 into the low buoyant density fraction (lanes C and D).

detergent insoluble fraction (figure 6; lane C) since the majority of bound antibody remained in the high-density portion of the buoyant density gradient. However crosslinking of bound mAb 7H.3 with rabbit anti-mouse polyclonal antibody induced a dramatic redistribution of mAb 7H.3 into the low buoyant density lipid raft component suggesting that aggregation of MHC class II into a large multimeric complex is sufficient to induce reorientation of surface MHC class II into the lipid raft domain (Figure 6; Lane D). Redistribution of anti-MHC class II mAb 7H.3 into the lipid raft did not alter the overall distribution of CD9 within the 1% CHAPS detergent insoluble fraction. This observation implies that CD9 can translocate into the lipid raft environment independently of MHC class II clustering. However, it does not rule out the possibility that CD9 influences access of MHC class II to the detergent insoluble membrane in the absence of crosslinking or that CD9 alters the immediate signalling environment of MHC class II within the lipid raft. The inability to demonstrate co-distribution of CD9 into the lipid raft following MHC class II crosslinking is puzzling. One possibility is that CD9 dissociates from MHC class II on entering the lipid raft and is targeted to an endocytic vesicle destined for degradation. HLA-DM expression is correlated with the appearance of empty MHC class II heterodimers on the plasma membrane. Empty MHC class II/HLA-DM complexes have a short half-life at the plasma membrane and are thought to be cleared through an endocytic mechanism [15].

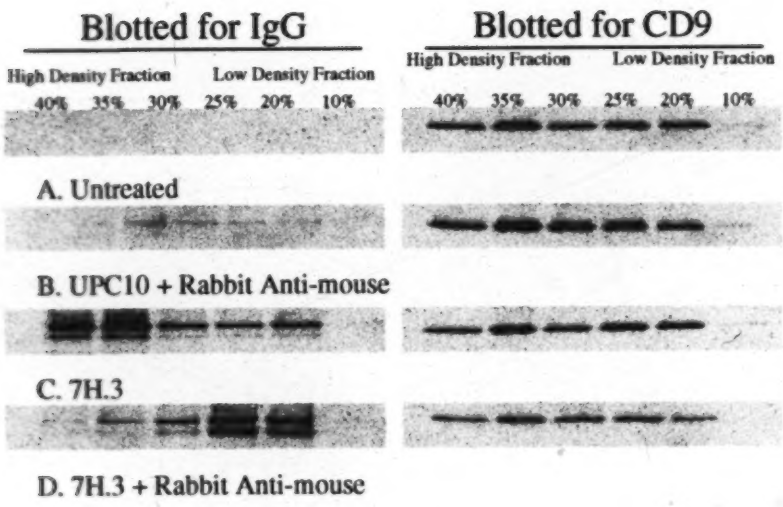

Figure 6. *Cross-linking 7H.3-bound MHC class II on the surface of Raji/CD9 transfectant cells induces translocation of 7H.3 into the low buoyant density fraction in the CHAPS detergent insoluble fraction independent of CD9.* Raji/CD9 transfectant cells are treated with anti-MHC class II mAb 7H.3 for 60 min at 4°C, followed by treatment in the presence or absence of rabbit anti-mouse polyclonal Ab for 60 min at 37°C. Cells were then lysed in 1% CHAPS, subjected to buoyant density centrifugation, then fractionated sample proteins were separated under continuous 5-20% non-reducing PAGE conditions. Proteins are resolved by ECL. MHC class II ligated mAb 7H.3 remains appears in the high density fractions of the 1% CHAPS detergent insoluble fraction (lane C) however following addition of secondary cross-linking antibody translocates to the low density component (lane D). The state of MHC class II antibody ligation does not alter the distribution of CD9 between high and low density components of the detergent insoluble fraction.

3.3.2. Does CD9 affect the proportion and distribution of MHC class II molecules resident within the detergent insoluble fraction?

Given the ability of either CD9 or MHC class II to associate with lipid rafts in response to antibody induced clustering, we asked whether CD9 might regulate access of MHC class II to components of the detergent insoluble fraction. In order to evaluate the role of CD9 in modulating access of MHC class II to the detergent insoluble fraction, Raji and Raji/CD9 cells were studied under equivalent conditions of antibody ligation. Here, cells were left untreated or exposed to anti-MHC class II (4^0C, 60min) followed by crosslinking with rabbit anti-mouse polyclonal antibodies (37^0C, 20min). Following Dounce homogenization, post nuclear fractions were separated by differential centrifugation. The detergent insoluble post nuclear membrane fraction was extracted in 1% CHAPS detergent and separated on a 10-40% discontinuous sucrose gradient. Gradient fractions were TCA precipitated and analysed by non-reducing PAGE. Under these conditions the detergent insoluble MHC class II fraction distributes between lipid rafts (low-buoyant density) and intermediate density fractions. These intermediate fractions may contain both cytoskeletal and lipid raft components as digestion of the actin cytoskeleton results in a redistribution of material into higher density fractions. Untreated, Raji/CD9 cells retained a significantly larger pool of MHC class II compared to non-transfected Raji within the 1% CHAPS detergent insoluble fraction (Figure 7; lanes C and A respectively). The increased retention of MHC class II in CD9 transfected cells suggests that CD9 may regulate access and/or retention of MHC class II within the detergent insoluble membrane.

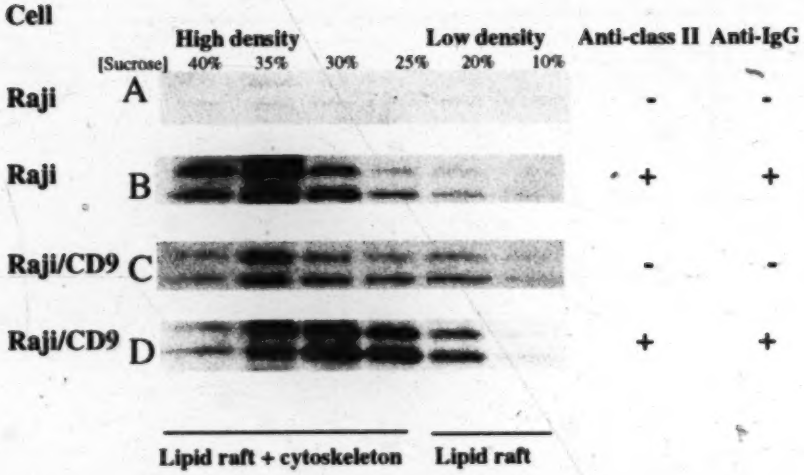

Figure 7. *MHC class II is a constitutive member of the Triton-insoluble fraction in Raji/CD9 transfectants but not in Raji, and translocates into the Triton-insoluble fraction on clustering of MHC class II with first and second antibody in both Raji and Raji/CD9.* Raji and Raji/CD9 cells were either left untreated or exposed to anti-MHC class II mAb 7H.3 followed by anti-mouse IgG. Cells are then lysed by Dounce homogenization and the post nuclear fraction (PNF) is obtained by differential centrifugation. The PNF is extracted in 1% CHAPS under cytoskeleton-stabilizing conditions, subjected to buoyant density centrifugation, and fractionated samples separated by continuous 5-20% non-reducing PAGE. Under these conditions the detergent insoluble material distributes throughout the gradient because the actin cytoskeleton and the lipid raft remain associated. The rationale for performing the experiment this way rather than by standard application of the entire lysate is to differentiate in the higher density fraction between the MHC class II that is associated with raft/cytoskeleton and MHC class II that is detergent soluble. SDS-PAGE analysis and blotting with a polyclonal anti-MHC class II antibody demonstrates that the level of MHC class II associated with the insoluble fraction is enhanced by CD9 expression. Data collected in Figure 7 was prepared by Alan Mak and is representative of results obtained from other experiments.

Interestingly, the detergent insoluble MHC class II pool retained within resting Raji/CD9 transfectant cells appears to be largely associated with the higher-density fractions. Retention of MHC class II within the high-density component of the detergent insoluble fraction may indicate that retention involves cytoskeletal elements as cytoskeletal associated proteins such as tallin and paxillin typically blot within these same high density fractions (Andrew Shaw, personal communication).

MHC class II crosslinking translocates a large pool of MHC class II into the detergent insoluble fraction in both non-transfected and Raji/CD9 transfected cells. Consistent with earlier observations (Figure 6), MHC class II is able to enter the detergent insoluble fraction in the absence of CD9 expression when it is cross-linked. The ability of MHC class II to translocate into low buoyant density fraction, however, is influenced by expression of CD9 as more MHC class II is present within the low buoyant density fractions from Raji/CD9 transfectant cells.

Section 3.4. Association of MHC class II with the cortical actin cytoskeleton

3.4.1. Does the distribution of MHC class II alter the dynamics of the cortical actin cytoskeleton?

Co-capping experiments involving the TCR in T-lymphocytes have revealed that clustering results in local thickening of the actin cytoskeleton under regions of TCR-lipid raft accumulation. Whether MHC class II clustering can direct similar

reorganization of cortical actin remains controversial. Here, we asked whether MHC class II clustering in regions of detergent insolubility could direct local thickening of the actin cytoskeleton under conditions of antibody ligation. Given the potential for CD9 in untreated cells to direct access of MHC class II to the detergent insoluble fraction, we also investigated whether CD9 expression could influence MHC class II regulation of actin thickening. MHC class II directed actin accumulation was analysed by confocal microscopy using the detergent extraction method of Geppert and Lipsky [57] in the presence (Raji/CD9) or absence (Raji) of CD9 expression. F-actin accumulation was visualised through phalloidin-FITC staining following detergent extraction. The role of lipid rafts in MHC class II induced actin reorganization is assessed by comparison of localized actin accumulation with detergent insoluble MHC class II accumulation.

Raji and Raji/CD9 cells were treated in parallel with directly fluorescent conjugated anti-MHC class II (mAb 7H.3-Alexa 594) on ice for 45 min. Following surface labelling cells were warmed to 37^{0}C in the presence or absence of rabbit anti-mouse polyclonal crosslinking antibodies for 45 min. Cells were then treated with 0.5% Triton X-100 supplemented CSB to extract detergent soluble material. Treatment of cells with 1% Triton X-100 compromises the structural integrity of the cells resulting in fragmentation, however, at 0.5% Triton X-100 cells remain intact allowing for immunofluorescence microscopy. Following extraction cells are fixed and stained for cortical actin with phalloidin-FITC. Analysis by confocal microscopy reveals that MHC class II is enriched in the detergent insoluble material following transfection of

CD9 in non-crosslinked cells (Figure 8; A-C (Raji) and D-F (Raji/CD9)). Crosslinking MHC class II invokes translocation of a large portion of MHC class II into the detergent insoluble fraction in both CD9 transfectant and non-transfectant cells (Figure 8; G-I (Raji) and J-L (Raji/CD9)) visualized as a capped region of intense staining. As demonstrated by phalloidin-FITC staining, enriched regions of cortical actin underlie regions MHC class II capping (Figure 8; G-I (Raji) and J-L (Raji/CD9)). This observation suggests that MHC class II clustering within detergent insoluble regions parallels local thickening of the actin cytoskeleton.

MHC class II regulation of actin reorganization could occur as a result of local increases in polymerization with or without concurrent de-polymerization in other regions of the actin cytoskeleton. Flow cytometry analysis of phalloidin-FITC stained cells following MHC class II crosslinking indicate that elaboration of the actin cytoskeleton under regions of MHC class II capping does not parallel overall increases in the pool of polymerized actin. Preliminary experiments revealed an overall decrease in F-actin staining of ~30% after cosslinking 7H.3 bound MHC class II with rabbit anti-mouse polyclonal Abs on the surface of Raji (Appendix, Figure 26). Lack of increased cellular pools of polymerized actin indicates that MHC class II is modulating reorganization of the entire actin cytoskeleton rather than simply increasing local *de novo* polymerization. In contrast, increased local *de novo* polymerization without comparable de-polymerization in other regions would lead to a shift in equilibrium from monameric to polymerized cellular actin and would be evident as an overall increase in phalloidin-FITC staining.

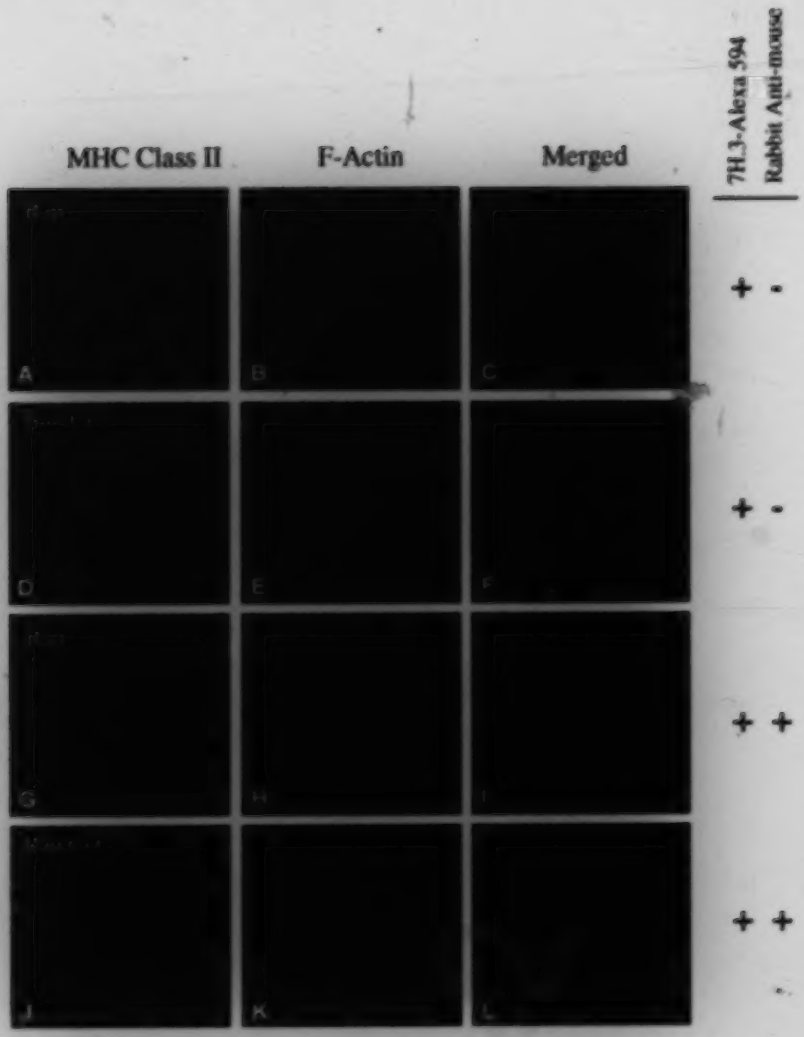

Figure 8. *MHC Class II retention within the Triton X-100 insoluble fraction of Raji and Raji/CD9 transfectant cells.* Raji (A-C, G-I) and Raji/CD9 (D-F, J-L) were incubated with anti-MHC Class II mAb (7H.3-Alexa 594) for 60 min on ice followed by 60 min at 4°C in the presence or absence of secondary rabbit anti-mouse IgG (G-L) cross-linking antibody. Cells were then warmed to 37°C for 45 min after which they extracted into 0.5% Triton X-100 for 5 min at 4°C to remove soluble membrane components, then fixed and stained with Phalloidin-FITC. Triton X-100-insoluble MHC Class II is retained in both Raji and Raji/CD9 following secondary antibody induced cross-linking (G and J). Cells treated with mAb 7H.3-Alexa 594 alone retained MHC class II bound 7H.3 on the surface of Raji/CD9 (D) but not Raji (A). Phalloidin-FITC staining of the actin cytoskeleton reveals comparable levels of F-actin organization in Raji (B and H) and Raji/CD9 (E and K). Local accumulation of F-actin is observed under regions of MHC class II cross-linking (E and K). Merged images; C,F,I,L.

Given the ability of CD9 to modulate the residency of MHC class II in the detergent insoluble fraction (Figure 7), we investigated the ability of CD9 expression to regulate β1-integrin entrance into the same fraction under similar conditions of antibody ligation. Raji B-cells express adhesion receptors of the β1-integrin family that can associate with CD9 in lymphocytes [86, 94]. Previously our lab has identified β1-integrin as a surface receptor that through antibody-dependent crosslinking can be induced to enter into the 1% CHAPS and 1% Triton X-100 detergent insoluble raft similar to MHC class II. Here, Raji and Raji/CD9 transfectant cells were treated with anti-β1-integrin mAb AP1.138 at 4^0C for 60 min followed by warming to 37^0C in the presence or absence of rabbit anti-mouse polyclonal crosslinking antibodies for 45 min. Following clustering at 37^0C, cells were lysed in CSB supplemented with 0.5% Triton X-100 as described previously and analyzed by laser scanning confocal microscopy. Unlike MHC class II, neither Raji nor Raji/CD9 cells were capable of retaining labelled β1-integin on the surface of extracted cells in the absence of crosslinking (Figure 9; A-C (Raji) and D-F (Raji/CD9)). This result indicates that the increased access to the detergent insoluble fraction observed following expression of CD9 is specific to MHC class II. Antibody induced crosslinking of β1-integrin on the surface induced local patches of detergent insoluble retention, but failed to generate large cap-like structures or local thickening of the actin cytoskeleton (Figure 9; G-I (Raji) and J-L (Raji/CD9)) observed with MHC class II.

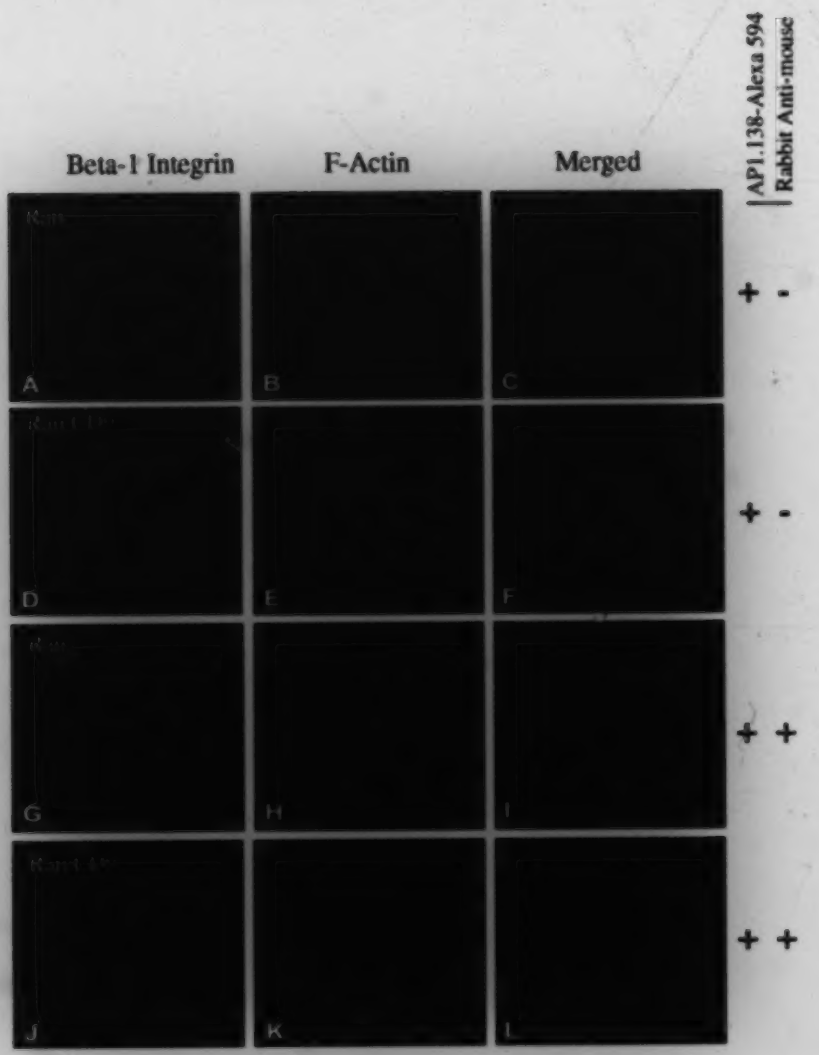

Figure 9. *Analysis of β1-integrin retention within the Triton X-100 detergent insoluble fraction in Raji and Raji/CD9 transfectant cells.* Raji (A-C, G-I) and Raji/CD9 (D-F, J-L) transfectant cells were incubated with anti-β1 integrin mAb AP1.138-TRITC for 60 min on ice followed by an additional 60 min in the presence or absence of rabbit anti-mouse IgG (G-L). Following labeling cells were warmed to 37°C for 45 min to initiate clustering. As described in Figure 8, cells were subjected to detergent extraction followed by F-actin staining with phalloidin-FITC (B,E,H, and K). In both Raji and Raji/CD9 transfected cells retention of β1-integrin within the 0.5% Triton X-100 detergent insoluble fraction was evident only after addition of secondary cross-linking antibody. The lack of β1-integrin retention following exposure to mAb AP1.138 alone indicates that CD9 expression does not alter its retention within the 0.5% Triton X-100 detergent insoluble fraction (A and D).

3.4.2. Are lipid rafts required for increased retention of MHC class II in the detergent insoluble fraction/actin cytoskeleton?

Traditionally, agents capable of extracting cholesterol from the membrane of live cells have been used to study lipid raft function. Removal of cholesterol is thought to result in dissolution of raft components by altering the physical environment within the membrane. Here, we have employed the cholesterol sequestering agent Methyl-β-Cyclodextrin (MBCD) to investigate the contribution of lipid rafts to CD9 enhanced MHC class II retention within the detergent insoluble fraction.

Raji and Raji/CD9 cells were precultured in the presence or absence of 5 mM MBCD for 60 min prior to labelling with fluorescent conjugated anti-MHC class II mAb 7H.3-Alexa 594. Again cells were extracted into 0.5% Triton X-100 supplemented CSB, fixed and stained for F-actin. Analysis of label retention reveals that removal of cholesterol prior to antibody ligation negates any enhanced MHC class II retention in the insoluble fraction observed following CD9 transfection (figure 10). Interestingly pretreatment of both Raji and Raji/CD9 resulted in a parallel decrease in polymerized F-actin as observed by a decline in phalloidin-FITC staining intensity. Under identical conditions of MBCD treatment, cells incubated for 90 min then assessed by trypan blue staining revealed 96% viability ruling out cell death as a contributor to the decrease in observed actin staining (Allan Mak, personal communication). Flow

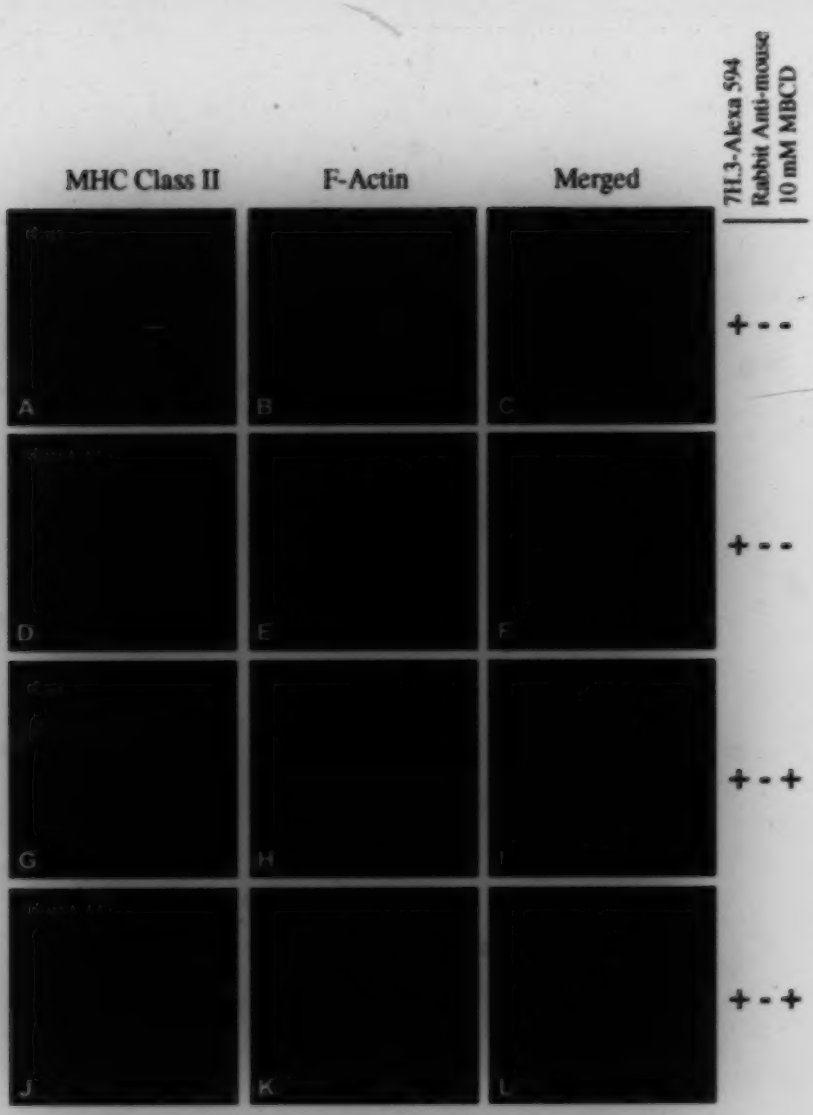

Figure 10. *Effect of cholesterol depletion on MHC class II retention within the Triton X-100 detergent insoluble fraction.* Raji (A-C, G-I) and Raji/CD9 (D-F, J-L) transfectant cells were pretreated with (G-L) or without (A-F) 5 mM methyl-β-cyclodextrin (MBCD) for 60 min. Cells were then incubated with anti-MHC class II mAb 7H.3-Alexa 594, extracted into 0.5% Triton X-100 under cytoskeletal stabilizing conditions and stained with phalloidin-FITC as described in figure 8. The enhanced association of MHC class II with the detergent insoluble fraction of Raji/CD9 transfectant cells was inhibited by MBCD sequestration of cholesterol (D (untreated) versus J (treated)). In addition MBCD pretreated cells show reduced pools of polymerized actin as indicated by diminished phalloidin-FITC staining (I and L).

cytometric analysis of phalloidin-FITC staining following 5 mM pretreatment with MBCD indicate a reduction of ~ 60% in both Raji and Raji/CD9 transfectant cells (Figure 11). The observed effect of MBCD on overall cytoskeletal staining suggests that lipid rafts play a pivotal role in cortical actin maintenance. Furthermore, this result indicates that the ability of CD9 to promote an association between MHC class II and the Triton X-100 detergent insoluble fraction may occur downstream of entrance into lipid rafts and involve stimulation of actin polymerization.

3.4.3. Is cortical actin polymerization required for retention of MHC class II within the detergent insoluble fraction?

In order to examine the role of the actin cytoskeleton in maintaining association of MHC class II and CD9 in the detergent insoluble fraction, we employed the sea urchin metabolite latrunculin A, a potent inhibitor of actin polymerization [110, 111]. Here we asked whether disruption of cortical actin polymerization altered the resident pool of CD9 or MHC class II within the insoluble fraction. Using flow cytometry as a read-out for retention of ligated CD9 or MHC class II within the detergent resistant fraction, cells were extracted into CSB + 0.5% Triton X-100 as described previously. Following extraction the remaining label retained within the insoluble material was analyzed by flow cytomemtry and quantified as the mean fluorescence. Pretreatment of cells with 0.5 μM latrunculin A for 90 min prior to antibody ligation resulted in a ~ 5-fold decrease in both MHC class II and CD9 retention in the Triton X-100 detergent insoluble (Figure 12 and 13 respectively). This indicates that active actin

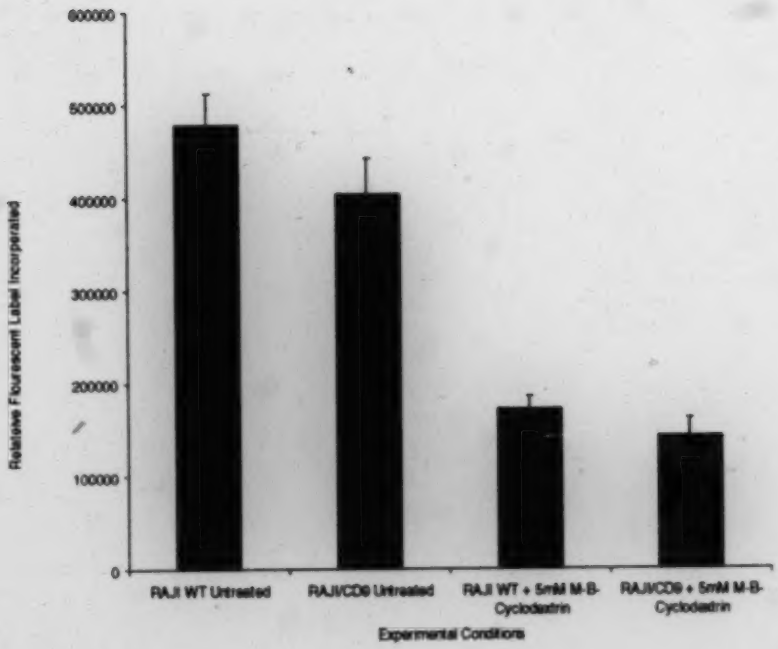

Figure 11. *Effect of Methyl-β-Cyclodextrin pre-treatment on maintenance of the cortical actin cytoskeleton in Raji and Raji/CD9 transfectant cells.* Cells were pretreated with or without 5 mM M-β-Cyclodextrin for 90 min at 37°C. Cells were then fixed, permeabilized by a brief 5 min extraction in 0.5% Triton X-100, followed by staining with phalloidin-FITC. Analysis of cortical actin staining intensity is performed by 3-dimensional analysis of confocal images using Bitplane Imaris 3-dimentional imaging software as described in materials and methods. Pre-treatment of both Raji and Raji/CD9 decreased phalloidin-FITC staining by ~50% indicating that fewer sites for phalloidin-FITC binding are available.

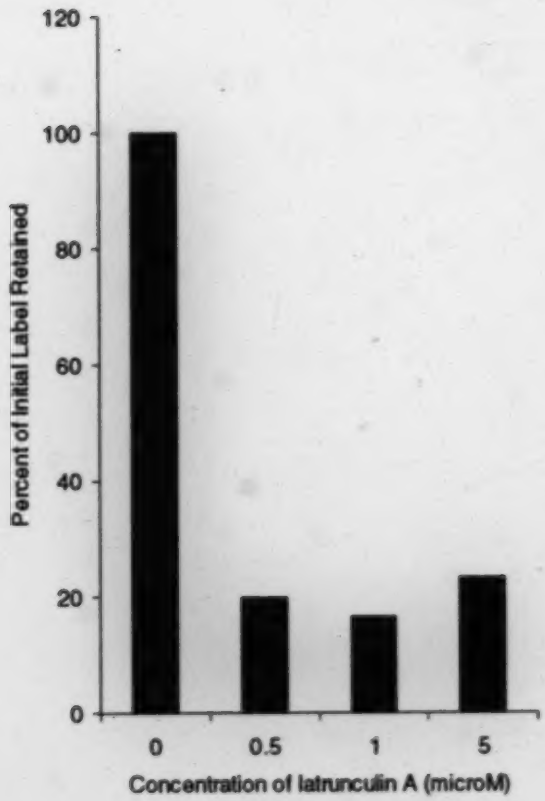

Figure 12. *Retention of MHC Class II in the Triton-X100 detergent insoluble fraction following disruption of actin polymerization with Latrunculin A.* Raji/CD9 cells were pretreated with increasing concentrations of latrunculin A. Cells were then incubated with anti-MHC Class II mAb 7H.3-Alexa 488, extracted into 0.5% Triton X-100 and fixed as described for figure 8. Cells were then analyzed by flow cytometry as described in materials and methods. Pretreatment of Raji/CD9 with latrunculin A, an inhibitor of actin assembly, diminishes retention of MHC class II within the Triton X-100 detergent insoluble fraction.

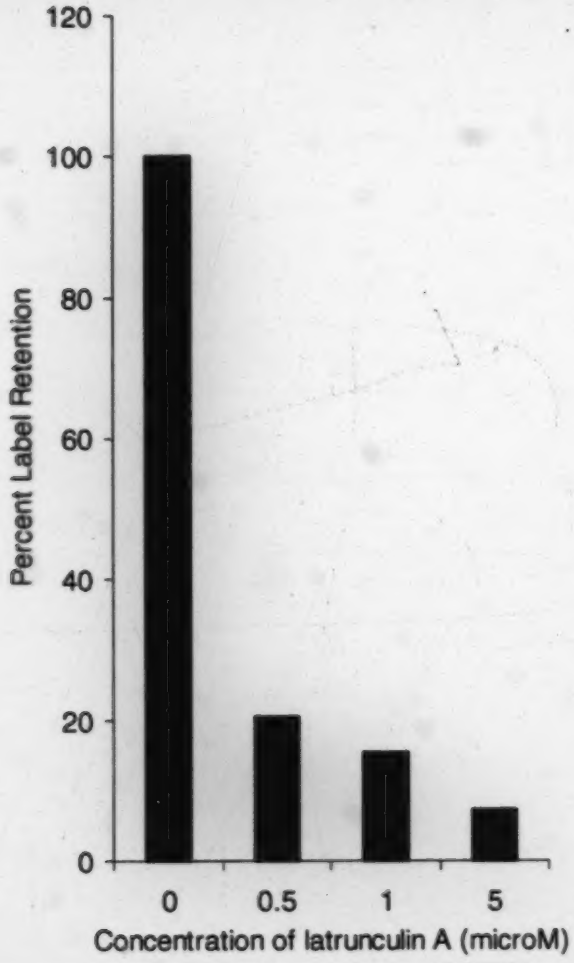

Figure 13. *Retention of CD9 in the Triton-X100 detergent insoluble fraction following disruption of actin polymerization with Latrunculin A.* Raji/CD9 cells were pretreated with increasing concentrations of latrunculin A for 60 min at 37°C. Cells were then labeled with anti-CD9 (mAb 50H.19-Alexa 488). Following antibody labeling cells were extracted into 0.5% Triton X-100 then fixed. Cells were then analyzed by flow cytometry as described in materials and methods. As observed for MHC class II, latrunculin A pretreatment of Raji/CD9 diminishes retention of CD9 within the Triton X-100 detergent insoluble fraction.

polymerization may be a prerequisite for preservation of MHC class II and CD9 within the detergent insoluble membrane. In addition loss of retention provides strong evidence that MHC class II does form associations with polymerizing actin whether through associative proteins or direct physical interactions.

Section 3.5. Chapter Summary

In Raji B-cells, MHC class II and ectopically expressed CD9 are in close proximity on the plasma membrane. Colocalization of MHC class II and CD9 on the surface of 1% CHAPS or 0.5% Triton X-100 extracted cells indicates that both proteins reside within the similar regions of detergent insolublility on the surface of B-cells. Co-immunoprecipitation of CD9 with anti-MHC class II mAb directed against the surface pool of MHC class II in 1% CHAPS demonstrates that MHC class II and CD9 maintain a physical association on the plasma membrane. Interestingly, MHC class II cannot be reciprocally co-immunoprecipitated from an anti-CD9 immunoprecipitate suggesting that ligation of CD9 with mAb 50H.19 prevents CD9 from physically associating with MHC class II or the antibody is unable to recognize CD9 coupled to MHC class II. Since CD9 partially colocalizes with MHC class II on the cell surface it is likely that the labelled CD9 is associated with MHC class II/CD9 complexes. Alternatively, MHC class II and CD9 are able to localize within similar regions of the plasma membrane independently of their association.

Analysis of CD9 and MHC class II distribution within the detergent insoluble fraction by buoyant density centrifugation on 10-40% sucrose gradients supports the observation that MHC class II and CD9 can partition into the 1% CHAPS detergent insoluble fraction independently of each other. Crosslinking of MHC class II in both Raji and Raji/CD9 results in a dramatic increase in the proportion of MHC class II within the insoluble fraction. This observation suggests that MHC class II, upon antibody induced clustering, can access the detergent insoluble fraction independent of CD9. However, transfection of CD9 is sufficient to increase the proportion of MHC class II retained within the detergent insoluble fraction in uncross-linked Raji B-cells suggesting that CD9 does promote association under conditions of minimal receptor clustering. Interestingly, CD9 crosslinking did not increase the association of MHC class II with the detergent insoluble fraction nor did it effect the distribution of existing MHC class II between lipid rafts and cytoskeletal components within the insoluble fraction. This suggests that MHC class II translocation into the detergent insoluble fraction is promoted by crosslinking and further enhanced by expression of CD9.

The contribution of actin cytoskeleton association and lipid rafts to the retention of MHC class II within the detergent insoluble fraction was investigated using the extraction technique of Geppert and Lipsky (1991) that stabilizes the actin cytoskeleton. Removal of cholesterol with MBCD to disrupt raft function resulted in loss of the selective increase in MHC class II retention afforded by expression of CD9. Thus, CD9 requires intact lipid rafts in order to maintain the association of MHC class II within the detergent insoluble fraction. Since inhibitors of actin polymerization

reduce MHC class II and CD9 retention within the detergent insoluble fraction active actin polymerization is required to maintain both proteins within the detergent insoluble fraction. The requirement for active actin polymerization suggests that the CD9-lipid raft induced association of MHC class II with the detergent insoluble fraction results in anchoring to the cortical actin cytoskeleton. Thus our results support a role for both lipid rafts and cytoskeletal assembly in CD9-enhanced association of MHC class II with the detergent insoluble fraction.

Chapter 4: Results. CD9 Regulation of MHC class II Trafficking

Section 4.1. Chapter Introduction and Experimental Objectives

Functional studies of MHC class II antigen presentation have focused on trafficking through intracellular compartments and association with chaperone-like molecules. Through these experiments, it has become apparent that MHC class I and class II acquire antigen distinct in origin and physical features. MHC class I and class II molecules utilize conformational differences in the peptide-binding regions, physically separated peptide loading compartments, and distinct chaperone molecule associations to select appropriate peptides for presentation. The recognition that distinct peptide loading compartments (PLC) exist for MHC class I and MHC class II has drawn attention to functional consequences of MHC trafficking within the cell. Namely, what mechanisms govern access of surface MHC to endocytic vesicles and what requirements exist for targeting within the endosomal pathway? MHC class II peptide loading may occur in a number of compartments within the endocytic pathway. The route by which newly synthesized MHC class II gains access to distinct pools of peptides and compartments of the endosomal pathway is largely governed by invariant chain association. However, the machinery required to direct mature recycling MHC class II to components of the endocytic pathway remains unclear and may involve regulation by one or more chaperone-like proteins.

Newly synthesized MHC class II molecules gain access to PLCs by two clearly identified routes, the classical pathway and the alternative pathway. The classical pathway is dependent upon association of MHC class II with the invariant chain (Ii). Ii chain serves an initial chaperone function providing stability, directing folding, and guiding trafficking to the early endosomal compartment through sorting signals in the cytoplasmic domain. Despite initial delivery to the early endocytic compartment, peptide-loading by the classical pathway is thought to occur in a late endosomal/early lysosomal compartment (MIIC; MHC class II containing compartment). Delayed access to peptide while traversing the endocytic pathway is regulated through the MHC class II-associated invariant chain peptide (CLIP). Eventual displacement of CLIP from the peptide-binding region through the actions of acid proteases and two chaperone-like molecules, HLA-DM and HLA-DO, allow peptide loading. In contrast, the alternative pathway involves trafficking to the plasma membrane followed by uptake into the early endosomal compartment. Current opinion suggests that endocytosis is directed by signals residing in the cytoplasmic tail sequence of MHC class II heterodimers and the associated Ii chain. A third less characterized route of trafficking to PLC involves recycling surface MHC class II into the early endosomal compartment (VIIC; vesicular MHC class II containing compartment). Recycling MHC class II is able to deliver relatively un-processed peptide antigens to the plasma membrane and may act as a mechanism for early response to antigenic insult by directing rapid turnover of antigenic determinants on the cell surface. Internalisation of the majority of MHC class II is independent of Ii chain signals, however, it may involve other chaperone-like proteins on the plasma membrane.

To date, studies investigating endocytosis of mature MHC class II from the surface of APCs have been unable to identify regulatory molecules governing access to endocytic compartments. Furthermore, the contribution of MHC class II complex constituents remain to be evaluated in the context of MHC class II trafficking. The presence of CD82 in MIIC peptide-loading compartments combined with the observed participation of CD81 and CD9 in MHC class II complexes at the cell surface indicates that tetraspanin proteins may influence class II trafficking into the endocytic compartment. As such temporal regulation of CD9 could have profound effects on MHC class II antigen presentation.

PI 3-kinase has been implicated in maintenance of plasma membrane adhesion with the cortical actin cytoskeleton [112], the process of early endosomes fusion prior to microtubule motor driven transport [113], and membrane trafficking along the secretory and endocytic pathways [114, 115]. Furthermore, CD19 is capable of regulating downstream PI 3-kinase signals and is an inducible constituent of the CD81/D21/Leu^{-13} complex associated with MHC class II [116-118]. Added to this lipid rafts may act as a potential site of phosphoinositide synthesis, and as a result, through lipid rafts MHC class II may access an environment suited to lipid kinase regulation. As such lipid kinases represent a potential downstream effector involved in MHC class II trafficking.

One of the difficulties in studying internalization of surface receptors is the inability to distinguish between subcellular pools of receptor with high sensitivity. Previously,

pulse-chase experiments or analysis of ligand uptake by flow cytometry have been employed to follow receptor internalization. The sensitivity of the former technique relies primarily on the ability purge surface label or the accuracy of subcellular fractionation. Similarly, use of flow cytometry is limited by the inability to distinguish the subcellular location of stained receptors. Here, we have developed a laser scanning confocal microscopy technique to sensitively quantify an internal pool of labeled receptor in live cells. Furthermore by employing 4-dimensional imaging we eliminate invasive subcellular fractionation or surface label purging steps allowing for analysis of a single population of cells over time. *Here, using 4-dimensional confocal microscopy, we provide evidence that CD9 may modulate the rate of MHC class II internalization following antibody ligation. Furthermore, we show that MHC class II internalization following antibody ligation is sensitive to cholesterol depletion and is opposed by the actin cytoskeleton. Finally we provide evidence that lipid kinases may be involved in MHC class II uptake following antibody ligation.*

Section 4.2. Regulation of MHC class II vesicular uptake into the endocytic compartment

4.2.1. Does CD9 expression influence internalization of MHC class II from the surface of Raji mature B-cells?

As mentioned earlier the ability of mature MHC class II to recycle into the endocytic pathway may have a profound impact on classical MHC functions. Here, we asked

whether CD9 could influence the accessibility of MHC class II to the endocytic compartment following antibody ligation. Raji and Raji/CD9 transfectant cells were exposed to directly conjugated anti-MHC class II mAb 7H.3-Alexa 594 at 4^0C for 45 min. Following labelling cells were placed in a heated chamber flushed with CO_2 on the stage of a Zeiss Axiovert 510 laser scanning microscope. MHC class II internalization was then analysed by 4-dimensional laser scanning microscopy over a period of 45min. Internalized material is observed through accumulation of internal bodies of intense staining usually located within the perinuclear region which we believe to represent fused endocytic vesicles. Transfection of CD9 increased the rate of MHC class II internalization by ~ 3- to 4-fold by 45 min (figure 14). This observation suggests that CD9 is able to increase access of mature surface MHC class II to endocytic machinery.

The uptake of transferrin takes place in clathrin-coated pits. To determine whether CD9 affects clathrin-mediated endocytosis in general we compared the uptake of transferrin-FITC between Raji and Raji/CD9 transfectant cells. Using flow cytometry as a readout for transferrin receptor (TfR) mediated uptake, Raji and Raji/CD9 cells were exposed to 50 μg/mL of transferrin-FITC at 37^0C. Raji cells expressing CD9 showed no difference over wild type Raji in transferrin uptake over 45 min. Increased MHC class II internalization in Raji/CD9 indicates that CD9 may regulate access of MHC class II to endocytic machinery following antibody ligation. However, since TfR

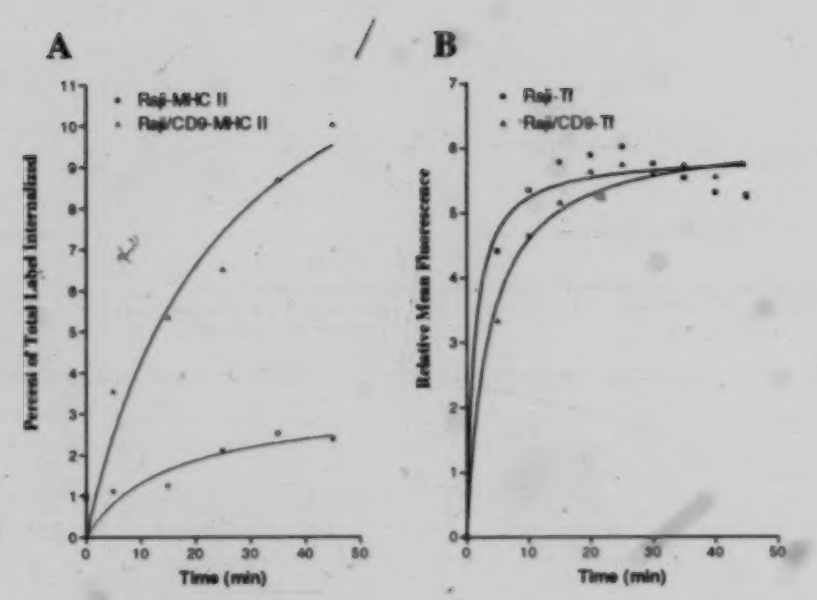

Figure 14. *Effect of ectopic CD9 expression in Raji B cells on the internalization of MHC class II and transferrin.* Internalization of MHC class II was followed in live cells by 4-dimensional confocal microscopy. In brief Raji and Raji/CD9 transfectant cells were pretreated on ice with anti-MHC class II mAb 7H.3-Alexa 594. Three dimensional images were generated from Z-stack series taken at intervals of 0.5 μm at 5 min and 45 min. Quantification of internalized label was analysed with Bitplane Imaris 3-D software. Internalisation of MHC class II is presented as percent of the average total label present on the series of cells. Transfection of CD9 increases the rate of MHC class II internalization by ~5-fold over 45 min (A). Uptake of transferrin-FITC was analyzed by flow cytometry in a Becton Dickson FACscan as described in materials and methods. In brief Raji and Raji/CD9 cells were exposed to 50 μg/ml of transferrin-FITC and aliquots were removed at given time points. Cells were then analyzed for mean fluorescence by flow cytometry. Internalization of unlike transferrin-FITC unlike MHC class II is not effected by transfection of CD9 (B).

uptake of transferrin-FITC remains unchanged following transfection the effect of CD9 on MHC class II is receptor specific. Despite the ability of CD9 to increase the rate of MHC class II internalization, CD9 itself does not appear to internalize following ligation of anti-CD9 mAb 50H.19-Alexa 488 on the cell surface (Figure 15). However, since mAb 50H.19 fails to co-immunoprecipitate MHC class II the antibody may not recognise CD9 when complexed to MHC class II. Alternatively, ligation of CD9 may alter access of CD9 to the endocytic machinery obscuring normal CD9 internalization when observed by confocal imaging.

4.2.2. Is internalization of MHC class II sensitive to cholesterol depletion with Methyl-Beta-Cyclodextrin?

Cholesterol has also been implicated in maintenance of the lipid raft environment and initiation of internalization by clathrin coated pit formation in epithelial cells [119, 120]. Observations in chapter 3 indicate that CD9 and MHC class II are able to access lipid rafts thought to be rich in cholesterol. This observation suggests that cholesterol may be a likely candidate for participation in MHC class II internalization. Given the established role of cholesterol in clathrin endocytosis and the ability of MHC class II to enter into lipid rafts we assessed the ability of MBCD to inhibit MHC class II internalization. Raji and Raji/CD9 cells were precultured in RPMI/BSA supplemented with 5 mM or 10 mM MBCD for 60 min at 37^{0}C. Following treatment cells were exposed to anti-MHC class II mAb 7H.3-Alexa 594 and prepared for 4-dimensional confocal analyses as described previously. Pretreatment resulted in a dose-dependent

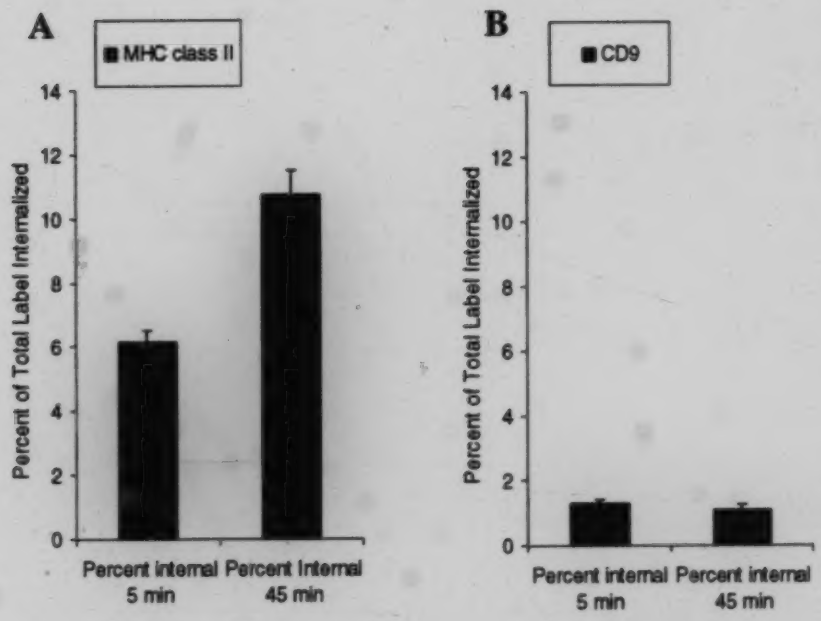

Figure 15. *Internalization of monoclonal antibody bound MHC class II and CD9 in Raji/CD9 transfectant cells.* Raji/CD9 transfectant cells were labeled with anti-MHC class II (mAb 7H.3-Alexa 594) or anti-CD9 (mAb 50H.19-Alexa 488). Internalization of antibody labeled MHC class II and CD9 is followed by 4-dimensional confocal microscopy using Bitplane Imaris 3-dimensional imaging software as described in Materials and Methods. MHC class II bound mAb 7H.3-Alexa 594 is internalized in Raji/CD9 transfectant cells (A). Under the same conditions CD9 bound mAb 50H.19-Alexa 488 internalization is negligible (B).

inhibition of CD9 enhanced MHC class II internalization (Figure 16). Pretreatment with 10 mM MBCD reduced the internalization of MHC class II in Raji from ~3.5% to <1% over the 45 min time period analysed. Raji/CD9 pretreatment with 10 mM MBCD decreased MHC class II internalization from >11% to <1% over the same period. MHC class II internalization in Raji/CD9 is therefore particularly sensitive to MBCD. The intermediate concentration of 5.0 mM MBCD reduced the rate of Raji/CD9 internalization by ~ 4-fold whereas 5.0 mM MBCD concentration decreased internalization by less than 2-fold in non-transfected Raji. In order to assess the sensitivity of transferrin-FITC uptake in Raji and Raji/CD9 following cholesterol depletion cells were precultured in MBCD under the same conditions described above prior to addition of 50 μM transferrin-FITC. Flow cytometric analyses indicate equivalent rates of internalization between Raji and Raji/CD9 at each of the MBCD concentrations analysed (Figure 17). Interestingly internalization of transferrin-FITC showed only moderate sensitivity to cholesterol depletion at the 5 mM MBCD concentration in both Raji and Raji/CD9 (~ 30%). The high sensitivity of Raji/CD9 to cholesterol depletion may therefore reflect the requirement of MHC class II to enter into the lipid raft component of the detergent insoluble fraction in order to access CD9 regulated endocytic machinery. Alternatively, cholesterol may be required for some function of the endocytic machinery accessed through CD9.

In order to assess whether the expression of CD9 alters the rate of fluid phase uptake, we asked whether BSA-FITC internalization differed between Raji and Raji/CD9 transfectant cells. BSA-FITC uptake was analysed by flow cytometry over a 105 min

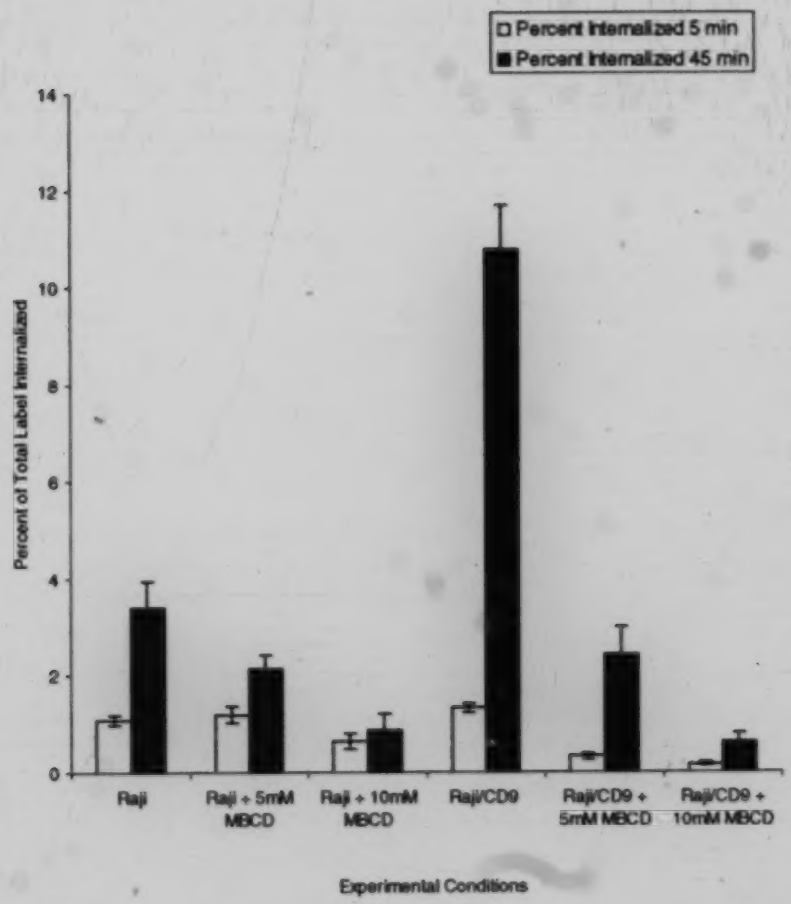

Figure 16. *Effect of cholesterol depletion on the internalization of mAb 7H.3 bound MHC class II in Raji and Raji/CD9 transfectant cells.* Raji and Raji/CD9 transfectant cells were preincubated in the presence or absence of increasing concentrations of MBCD. Cells were then labeled with anti-MHC class II mAb 7H.3-Alexa 594 and prepared for live cell 4-dimensional confocal microscopy to analyze MHC class II bound mAb 7H.3-Alexa 594 internalization as described in Materials and Methods. Pretreatment of both Raji and Raji/CD9 transfectant cells with MBCD decreased the rate of MHC class II bound mAb 7H.3-Alexa 594 internalization. Internalization of MHC class II in Raji/CD9 cells was particularly sensitive to cholesterol removal decreasing ~5-fold in the presence of 5 mM MBCD.

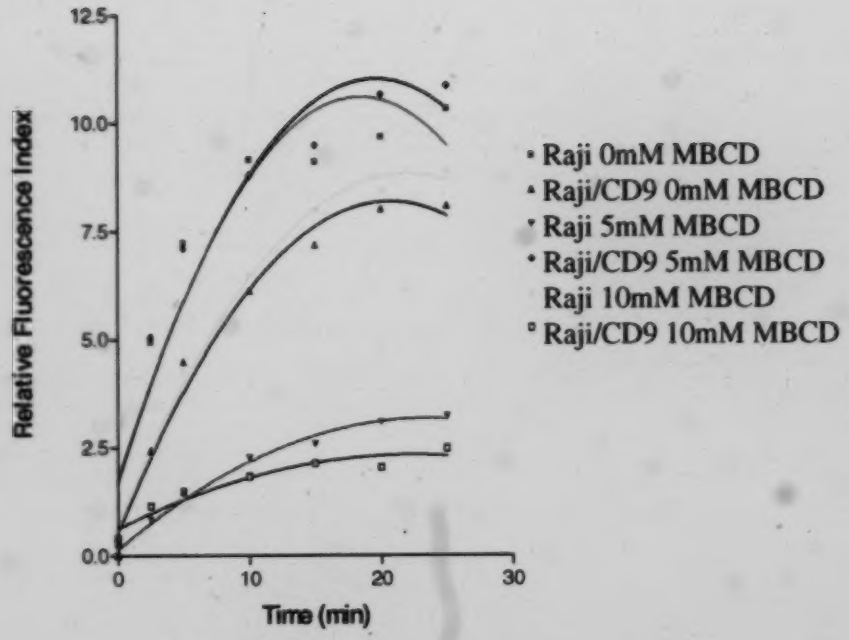

Figure 17. *Effect of cholesterol depletion on the internalization of transferrin-FITC in Raji and Raji/CD9 transfectant cells.* Raji and Raji/CD9 cells are harvested and preincubated with or without 5 mM to 10 mM MBCD for 30 min at 37°C. Cells were then supplemented with 50 µg/ml transferrin-FITC and aliquots were removed at specified time points. Transferrin-FITC uptake was quantified by flow cytometry as described in materials and methods. Internalization of transferrin is sensitive to 10 mM concentrations of MBCD but remains relatively unchanged by 5 mM MBCD.

period reflecting the slower kinetics of non-receptor mediated internalization of particulate material. Raji and Raji/CD9 cells were incubated in the presence or absence of 5 mM MBCD for 60 min as described for transferrin-FITC. Following cholesterol depletion samples were incubated with BSA-FITC at 37^{0}C. Expression of CD9 had no significant effect on the uptake of BSA-FITC (Figure 18) indicating that CD9 does not influence BSA uptake by fluid phase endocytosis. Pretreatment with 10 mM MBCD resulted in an approximately 50% reduction in the total uptake of BSA-FITC over 90 min. This level of inhibition shows similarity to the lower cholesterol depletion sensitivity observed for MHC class II in the absence of CD9 expression.

4.2.3. Is regulation of MHC class II internalization affected the underlying cortical actin cytoskeleton?

The actin cytoskeleton has been implicated in bi-directional vesicular fusion and trafficking at the plasma membrane. Internalization of tetraspanin family members, including CD63, is increased following disruption of the underlying actin cytoskeleton by agents which specifically inhibit actin polymerization. Similarly, vesicle fusion and release of neurotransmitter at post-synaptic junctions rely upon local permeation of the actin matrix to permit vesicular access to the plasma membrane. In order to dissect the role of polymerized actin in MHC class II endocytosis, we looked at the effect of actin disruption with cytochalasin D and latrunculin A. In brief, Raji and Raji/CD9 cells were incubated in the presence or absence of inhibitor for 60 min prior to labelling with fluorescent conjugated anti-MHC class II mAb 7H.3-Alexa 594. Internalization

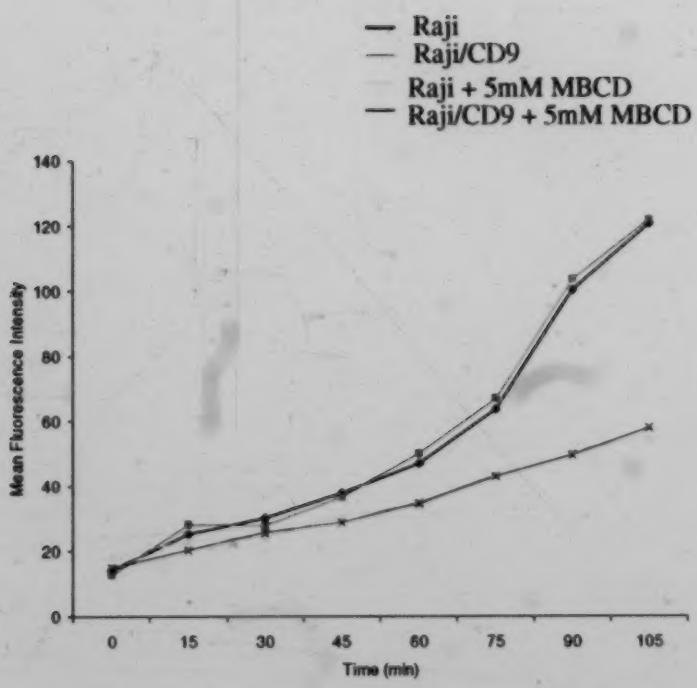

Figure 18. *Uptake of BSA-FITC in Raji and Raji/CD9 transfectant cells.* Raji and Raji/CD9 transfectant cells were incubated in the presence or absence of 5mM MBCD for 30 min at 37°C. Cells were then supplemented with 0.2% w/v BSA-FITC at time=0 min. Samples were removed at specified time points and BSA uptake was analyzed by flow cytometry to determine the mean fluorescence. Raji and Raji/CD9 cells show similar rates of internalization in both treated and untreated cells. Pre-treatment of cells with 5mM MBCD results in a ~ 50% decrease in the uptake of BSA-FITC by 90 min.

was followed as described previously using 4-dimensional laser scanning confocal microscopy. Disruption of the cortical actin cytoskeleton by pretreatment of cells with 1μM cytochalasin D increased MHC class II internalization in Raji from 3.0 +/- 0.3% standard error to 13.3 +/- 2.9% standard error over 45 min (Figure 19). Treatment of Raji/CD9 transfectants increased internalization from 14.5 +/- 1.9% standard error to 41.0 +/- 3.9% standard error over the same 45 min time period. The marked increase in the internalization of MHC class II on preventing actin polymerization strangely suggests that endocytosis is opposed by the actin cytoskeleton. To further substantiate the effect of blocking actin organization on MHC class II internalization, a second inhibitor of polymerization, latrunculin A, was employed under similar conditions. As with cytochalasin D, pretreatment of cells with latrunculin A for 60 min prior to labelling resulted in significant increase in the rate of mAb 7H.3-Alexa 594 bound MHC class II over 45 min (Figure 20). Raji increased from 2.9 +/- 0.3% standard error to 25.3 +/- 1.2% standard error whereas Raji/CD9 increased from 14.5 +/- 1.9% standard error to 28.7 +/- 3.0% standard error by 45 min. The increased rate of MHC class II internalization could result from decreases in the pool of polymerized actin. Alternatively, increases in the pool of monomeric actin may promote access of MHC class II to endocytic machinery by sequestering actin-binding proteins. Finally, by disrupting anchorage to the underlying cortical actin cytoskeleton MHC class II may be more readily incorporated into endocytic vesicles that bud off from the plasma membrane.

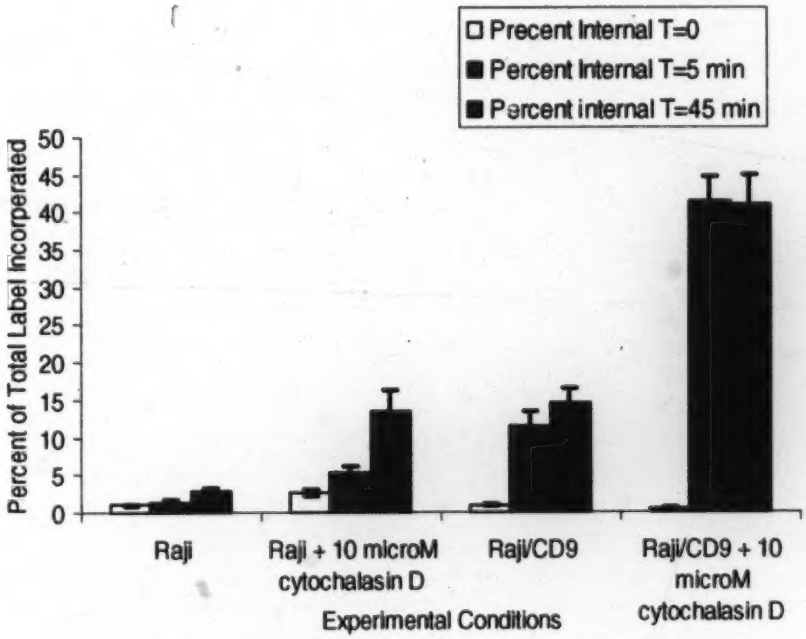

Figure 19. *Internalization of MHC class II following disruption of actin polymerization with cytochalasin D.* Raji and Raji/CD9 transfectant cells were preincubated in the presence or absence of 1 μM cytochalasin D for 60 min at 37°C. Cells were then labeled with anti-MHC class II (mAb 7H.3-Alexa 594), then resuspended with or without cytochalasin D. Internalization of MHC class II was analysed by 4-D confocal microscopy as described Materials and Methods. Pretreatment with cytochalasin D increases the rate of MHC class II bound mAb 7H.3 internalization by ~5-fold in Raji and ~3-fold in Raji/CD9 over 45 min.

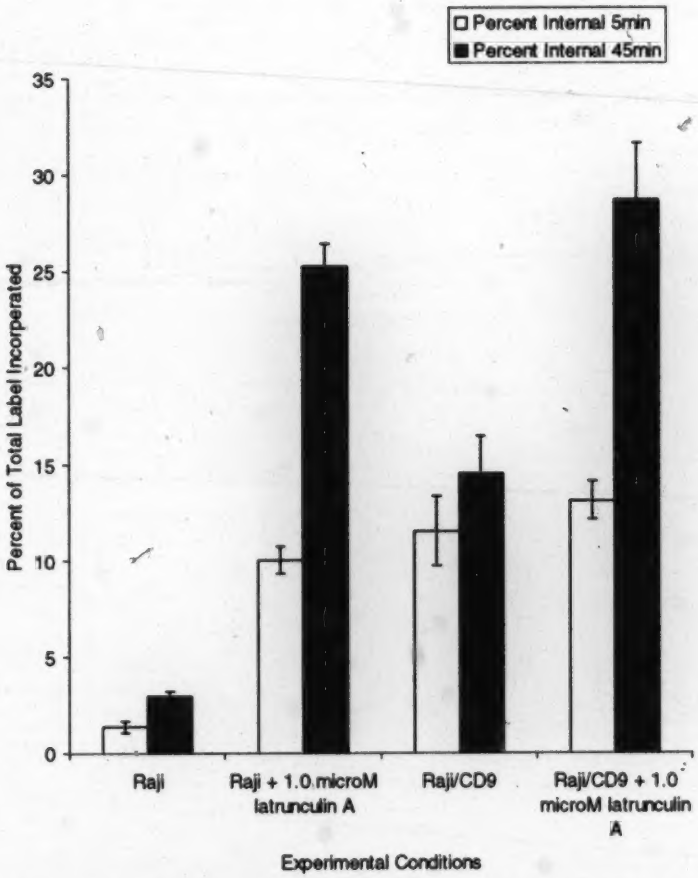

Figure 20. *Effect of latrunculin A inhibition of actin polymerization on the internalization of MHC class II in Raji and Raji/CD9 transfectant cells.* Cells were preincubated in the presence or absence of 2 μM Latrunculin A for 60 min at 37°C. Cells were then labeled with anti-MHC class II (mAb 7H.3-Alexa 594), then resuspended with or without Latrunculin A. Internalization of MHC class II was analyzed by 4-dimensional confocal microscopy as described in Materials and Methods. Pretreatment of Raji or Raji/CD9 transfectant cells with latrunculin A increased the internalization rate of MHC class II bound mAb 7H.3-Alexa594.

4.2.4. Is phosphoinositide metabolism involved in internalization of MHC class II following ligation with anti-MHC class II?

PI 3-kinases have been implicated in vesicle fusion [113] and trafficking [114, 115]. Through association with constituents of tetraspanin-MHC class II complexes such as CD19, PI 3-kinases may play a role in MHC class II internalization. We employed the PI 3-kinase inhibitors wortmannin and LY294002 to investigate a role for phosphoinositide signalling in MHC class II endocytosis following ligation with mAb 7H.3. Raji and Raji/CD9 cells were pretreated in the presence or absence of 1μM wortmannin or 10 μM LY294002. Pretreatment of Raji with wortmannin resulted in a ~ 50% decrease in internalization of MHC class II over 45 min from 3.7 +/- 0.1% standard error to 2.0 +/- 0.1% standard error (Figure 21; A). Raji/CD9 transfectant cells showed a more dramatic inhibition of ~10-fold with the percent of internalized signal falling from 10.7 +/- 0.1% standard error to 0.9 +/- 0.2% standard error (Figure 21; B). Interestingly, the PI 3-kinase inhibitor LY294002 failed to significantly inhibit MHC class II internalization in either Raji or Raji/CD9 transfectant cells. The differential ability of wortmannin and LY294002 to inhibit MHC class II internalization may implicate a different kinase in MHC class II internalization. However, the dramatic inhibition of MHC class II uptake in Raji/CD9 cells implicates a wortmannin sensitive mechanism in CD9 regulation of MHC class II access to the endocytic compartment. Introduction of lipid kinases to tetraspanin containing complexes may direct internalization machinery to MHC class II. Alternatively, CD9

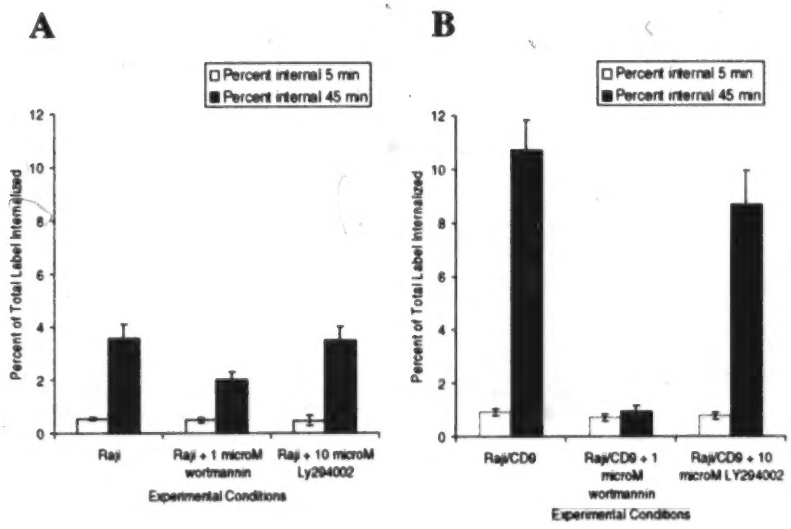

Figure 21 . *Effect of PI 3-kinase inhibitors Wortmannin and LY 294002 on the internalization of 7H.3-Alexa 594 ligated MHC class II in Raji and Raji/CD9 transfectant cells.* Raji and Raji/CD9 cells were pretreated 1 μM wortmannin or 10 μM LY 294002 for 60 min at 37°C. Cells were then stained with anti-MHC class II (mAb 7H.3-Alexa 594) and prepared as described in Materials and Methods for 4-dimensional confocal microscopy. Internalization of MHC class II bound mAb 7H.3-Alexa 594 is significantly inhibited in the presence of 1 μM wortmannin in both Raji (A) and Raji/CD9 (B). Pretreatment of cells with 10 μM LY 294002 had little effect on either Raji or Raji/CD9 internalization.

enhanced access of MHC class II to lipid raft may act to sequester MHC class II within a phosphoinositide enriched region of the plasma membrane.

4.2.5. Are Fc-receptors involved CD9 regulated internalization of MHC class II?

A limitation of using intact antibodies to follow protein trafficking in lymphocytes is the possible interaction of the Fc region with Fc receptors on the lymphocyte surface. Raji B-cells express a number of Fc receptors including FcγRIIa, FcγRIIb1 and FcγRIIb2 [121]. Co-ligation of FcγRIIb receptors have been shown to inhibit B-cell activation by interacting with components of the $p21^{ras}$-dependent pathway [122]. Recently, Wagle *et al.* demonstrated that FcγRIIb1 co-ligation through IgG coupled-antigen stimulation negatively regulated BCR-mediated internalization [114]. Fc receptors may similarly influence MHC class II function if co-engaged by anti-MHC class II mAb 7H.3 Fc regions. In order to investigate the potential role of Fcγ-receptors in MHC class II internalization from the surface of Raji B-lymphocytes, Fab fragments were generated from anti-MHC class II mAb 7H.3 and labelled with Alexa 594. Competitive binding of MHC class II by preincubation of Raji with excess concentrations of unlabelled intact mAb 7H.3 were sufficient to block Fab staining indicating that the Fab fragments generated retain ligand specificity. We next asked whether Fab bound MHC class II showed similar alterations in MHC class II internalization following CD9 expression. Analysis of Fab ligated MHC class II internalization by 4-dimensional confocal microscopy reveals that Raji and Raji/CD9 transfectant cells display equal rates of internalization over 45 min (Figure 22). The

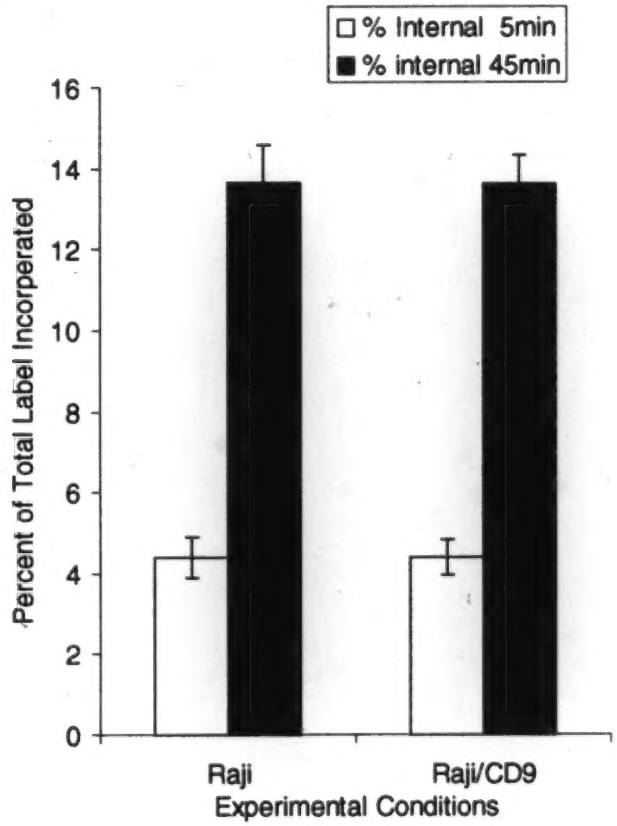

Figure 22. *Internalization of 7H.3-Alexa 594 Fab fragment bound MHC class II in Raji and Raji/CD9 transfectant cells.* Raji and Raji/CD9 cells are are labeled with 7H.3-Alexa 594 Fab fragments for 45 min at 4°C. Internalization of labeled Fab fragments is followed by 4-dimensional confocal microscopy as described in Materials and Methods. Unlike intact mAb 7H.3, Fab fragment-ligated MHC class II have equal rates of internalization in both Raji and Raji/CD9 transfectant cell. Equal rates of MHC class II bound 7H.3-Alexa 594 Fab fragment internalization irrespective of ectopic CD9 expression implicates the Fc receptor or antibody induced clustering as a negative regulator of MHC class II internalization in Raji B-cells.

internalized pool of Fab signal was 13.7 +/- 0.5% standard error and 13.6 +/- 0.4% standard error respectively for Raji and Raji/CD9 over the 45 min time period. The increased rate of Fab-ligated MHC class II internalization over intact mAb ligation in Raji indicates that a subset of Fcγ-receptors expressed on the surface of Raji may be down-regulating internalization of mAb bound MHC class II and that CD9 expression is sufficient to overcome the Fcγ-receptor mediated signal. Alternatively, in Raji the use of Fab fragments may prevent inhibitory signals for endocytosis generated through MHC class II dimerization that in a similar fashion could be overcome through CD9 expression. Interestingly pretreatment with mAb 7H.3 isotype-matched mAb UPC10 or an anti-FcγRIIa mAb IV.3 had no significant effect on the rate of MHC class II internalisation following addition intact mAb 7H.3-Alexa 594 .

Section 4.3. Chapter Summary

Introduction of CD9 into the mature B-cell line Raji results in an increase in the rate of mAb ligated MHC class II internalization into the endocytic compartment. The increased rate of internalization observed for MHC class II however does not appear to be a result of a common regulatory mechanism invoked through ectopic CD9 expression as neither BSA-FITC, a marker of fluid phase endocytosis, nor transferrin-FITC receptor mediated uptake were altered through CD9 expression. Depletion of cholesterol with 5 mM MBCD a cholesterol-sequestering agent resulted in partial inhibition of MHC class II, transferrin-FITC and BSA-FITC endocytosis. Dose response curves for MBCD inhibition however indicate that MHC class II

internalization is particularly sensitive to cholesterol depletion indicating that lipid raft may play an essential role CD9-mediated access to endocytic machinery following mAb 7H.3 ligation. Disruption of actin polymerization with cytochalasin D or latrunculin A results in marked increases in the rate of MHC class II internalization in both Raji and Raji/CD9 transfectant cells indicating that the actin cytoskeleton acts as a CD9-independent negative regulator of MHC class II internalization. Pretreatment of cells with nocodazol or colchicine, potent inhibitors of microtubule polymerization, failed to alter the internalization of MHC class II from the cell surface indicating that microtubular function may not be required for MHC class II uptake from the plasma membrane.

Investigation of the down-stream signals required for MHC class II internalization implicate PI 3-kinase or PI 4-kinase involvement as pretreatment with 1 μM wortmannin resulted in almost complete inhibition of MHC class II internalization in Raji/CD9 transfectant cells. Curiously, 10 μM LY294002 failed to significantly inhibit MHC class II internalization suggesting that a distinct LY294002-insensitive pool of PI 3-kinases may be implicated in the endocytic process governing class II internalization post antibody ligation. Use of Fab fragments indicates that CD9 expression overcomes negative signals in Raji following engagement of the Fc-receptor or alternatively MHC class II dimmer formation.

Chapter 5: Discussion

Recently, lateral segregation of molecules within the plasma membrane has been identified as a key feature in lymphocyte activation [73]. Lateral trafficking of cognitive (receptor) and effector (down stream signalling) molecules into distinct microdomains within the plasma membrane may coordinate formation of supra-molecular activation complexes (SMACs) and reorganization of the cortical actin cytoskeleton thereby providing local signal integration and sites for initiation of receptor trafficking into the endocytic compartment. A rapidly growing field of research into lipid raft function has indicated that the ability of proteins to segregate into regions of highly ordered lipid could alter their functional capacity. MHC class II and CD9 are associated on the surface of immature B-cells and have both been identified as components of the lipid raft. As such, the developmentally regulated tetraspanin CD9 is a good candidate for studying the regulation of MHC class II function within the lipid raft environment. Work designed to test these ideas, and described in this thesis, provide information on the role of CD9 and lipid rafts in coordinating MHC class II function on the plasma membrane of Raji B-cells.

T-cell activation following MHC class II presentation of foreign peptide requires sustained multivalent MHC class II-TCR engagement within the region of T-cell B-cell contact. How MHC class II is retained within a laterally distinct region of the B-cell membrane remains controversial. Early studies into MHC class II function have

indicated that MHC class II formed associations with the cortical actin cytoskeleton. However, identification of MHC class II within the lipid raft components of the detergent insoluble material questions the validity actin cytoskeleton association. Evidence provided in this study strongly implicates the cortical actin cytoskeleton in MHC class II retention within the detergent insoluble fraction since inhibition of actin assembly circumvents the retention of MHC class II within the detergent insoluble fraction and enhances endocytosis. These observations indicate that MHC class II association with the actin cytoskeleton requires *de novo* actin assembly and may occur through a lipid raft-dependent mechanism.

The interrelationship between lipid rafts and the cortical actin cytoskeleton have been suggested by studies into T-cell activation. Separate studies have indicated that TCR engagement results in local thickening of the cortical actin cytoskeleton [101] and aggregation of lipid rafts [123]. Our results extend upon previous studies that failed to stabilize the actin cytoskeleton [75]. Here, we demonstrate that the function of lipid rafts and the actin cytoskeleton may be coupled. Two lines of evidence support this model. First, cholesterol depletion inhibits MHC class II retention indicating that lipid raft may modulate MHC class II anchorage to polymerized actin. Furthermore, when cross-linked MHC class II enters into the low buoyant density component of the detergent insoluble fraction and by confocal microscopy is found to accumulate within detergent insoluble regions colocalized with regions of local actin cytoskeleton thickening. Second, cholesterol depletion results in decreased cellular pools of polymerized actin. We propose that MHC class II association with the cortical actin

cytoskeleton occurs downstream of entrance into the lipid raft and that conditions provided by the lipid raft environment favour local cytoskeletal remodelling necessary for anchorage to the cortical cytoskeleton. This model is supported by evidence that places lipid rafts in regions of the cell undergoing rapid cytoskeletal remodelling such as the leading edge of migrating lymphocytes. Perhaps not surprisingly then CD9, a tetraspanin involved in sperm and egg fusion as well as integrin mediated cell motility, has been identified as a major constituent of lipid rafts.

Ectopic expression of CD9 in mature Raji B-cells resulted in increased retention of MHC class II within the detergent insoluble fraction. Here, we extend the model proposed earlier to include CD9 as a regulator of MHC class II cytoskeletal association acting through a through a lipid raft dependent mechanism. We suggest here that association of CD9 with MHC class II within the soluble glycerolipid fraction of the plasma membrane alters the lipid affinity of the complex allowing it to enter into the highly ordered lipid raft environment (Figure 23). Conditions favouring actin cytoskeleton remodelling within the lipid raft promote MHC class II anchorage to the cytoskeletal matrix. The ability of MHC class II to enter into the detergent insoluble fraction and direct local actin assembly independently of CD9 following antibody crosslinking indicates that under conditions of strong stimuli MHC class II is able to either stimulate actin remodelling through a raft-independent mechanism or initiate raft involvement in the absence of CD9. Experiments designed to determine the location of lipid raft following MHC class II crosslinking could be used to test the later hypothesis. A commonly used label for localization of lipid raft is FITC conjugated

cholera toxin. As such cholera toxin-FITC could be used to test MHC class II regulated raft aggregation, however in our study Raji cells failed to show any significant staining indicating that the GM1 ligand is not expressed on our cell line.

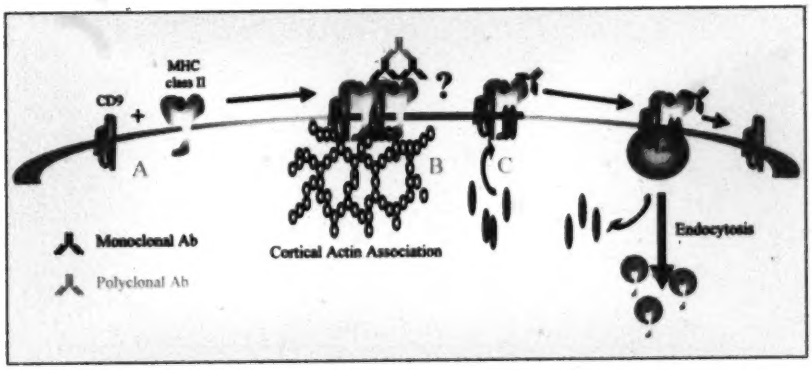

Figure 23. *A model of lipid raft regulated MHC class II association with the cortical actin cytoskeleton or initiation of endocytic complex formation.* Association of MHC class II with CD9 in the glycerolipid fraction of the plasma membrane (A) increases MHC class II residency in the lipid raft. The state of MHC class II activation by antibody ligation determines the balance between cytoskeletal association (B) and access to endocytic complexes (C).

Recent studies into lipid raft involvement in endocytosis have identified regions of lipid raft adjacent to sites of receptor internalization. The highly lipid ordered state of rafts make them unlikely candidates for direct involvement in vesicle uptake. However, lipid rafts may function as a reserve for cholesterol, implicated in clathrin-mediated endocytosis, or alternatively as a site for assembly of endocytic machinery components which then migrate into the surrounding glycerolipid. Following endocytosis MHC class II is present within endocytic compartments containing transferrin receptors. However, whether MHC class II enters into the endocytic compartment through the same clathrin-dependent mechanism as transferrin is not known. Investigation of CD9 involvement in MHC class II internalization events

reported here indicate that Raji/CD9 transfectant B-cells have increased rates internalization over Raji. Expanding further on the model proposed earlier, we suggest that CD9 directs MHC class II into an environment suitable for both initiations of vesicle uptake or attachment to the cortical actin cytoskeleton (Figure 23). In addition, we propose that the decision to enter into an endocytic vesicle or associate with the actin cytoskeleton is dependent upon the state of receptor activation and occurs within the raft environment. Evidence supporting this model and presented in this thesis are summarized as follows:

1) CD9 increases the proportion of MHC class II within both the lipid raft and cytoskeleton components of the detergent insoluble fraction.

2) Disruption of the actin cytoskeleton results in dramatic increases the rate of MHC class II internalization following antibody ligation suggesting that the cortical cytoskeleton is opposing endocytosis and that disruption of actin assembly favours endocytosis.

3) CD9 enhanced MHC class II internalization shows high sensitivity to cholesterol depletion over and above transferrin endocytosis. Clathrin-mediated endocytosis is sensitive to high concentrations of M-β- cyclodextrin concentration, however the elevated cholesterol sensitivity of MHC class II internalization indicates that MHC class II may require entrance into lipid raft prior to engaging the endocytic machinery.

4) Antibody crosslinking, which favours MHC class II association with the cortical actin cytoskeleton, greatly reduces MHC class II internalization whereas Fab ligation results in high rates of MHC class II uptake in both Raji and Raji/CD9 indicating that within the lipid raft environment the decision to enter into endocytic vesicles may depend on the state of receptor aggregation. In Raji/CD9 mAb ligation of MHC class II could represent an intermediate level of receptor activation that in the absence of CD9 expression favours cortical actin association. Alternatively, co-engagement of FcγRIIb receptors on the surface of Raji B-cells may inhibit MHC class II receptor endocytosis as observed for B-cell receptor internalization. Should this be the cause then CD9 overcomes the negative FcR signal.

5) Inhibition of PI 3-kinases by wortmannin blocks internalization of MHC class II following antibody ligation. Recent evidence suggests that tetraspanin family members may regulate lipid kinase activity. Furthermore, lipid rafts have been implicated as potential storage sites for phosphoinositides that have been implicated in membrane fusion events and membrane attachment to the actin cytoskeleton. CD9 expression, by increasing access of MHC class II to sites of phosphoinositide signalling, may influence MHC class II actin cytoskeleton association or entrance into the endocytic compartment.

The conditions directing MHC class II association with the actin cytoskeleton or endocytic machinery will require further investigation. Our observations indicate that the decision may depend on the state of MHC class II engagement as crosslinking MHC class II significantly slows internalization. The use of $(Fab)_2$ fragments would indicate whether the inhibition of MHC class II internalization observed in mAb 7H.3 ligated Raji is the result of FcR engagement or is mediated through antibody induced dimerization of MHC class II.

MHC class II antigen presentation must be tightly regulated during B-cell development and initiation of immune response to invading pathogens. Insight into the effect of MHC class II trafficking between components of the plasma membrane and the endocytic compartment will be essential to understanding fine control of MHC class II classical and non-function. To date, the regulation of mature MHC class II by constituents of MHC class II macromolecular complexes remains largely uninvestigated. Similar to the involvement of chaperone-like proteins in MHC class II maturation and peptide loading, participants of MHC class II containing complexes may alter mature MHC class II function. Evidence provided in this work suggests that the tetraspanin molecule CD9 through a chaperone-like function regulates mature MHC class II trafficking at the cell surface. Experiments designed here provide the first direct evidence that tetraspanin proteins may regulate the function of MHC class II through regulation of lateral trafficking within the plasma membrane. As such temporal regulation of CD9 expression may profoundly alter B-cell responses to receptor engagement. If the model proposed here is correct, expression of CD9 in

immature B-cells may provide a means of accelerating peptide loading and increasing MHC class II sensitivity to TCR engagement through entrance into lipid rafts. This accelerated function may help drive clonal selection by increasing B-cell sensitivity to negative selection pressures. Re-induction of CD9 expression following activation could drive proliferative events in antigen challenged B-cells. By decreasing the threshold of stimulus required for MHC class II to access detergent insoluble components of the plasma membrane, raft dependent phosphotyrosine signalling may be up regulated. Whether other members of the tetraspanin family are involved in MHC class II trafficking remain to be addressed. However, identification of CD82, CD151 and CD63 within MHC class II containing endocytic compartments and CD81 within complexes at the cell surface could point to a role for specific tetraspanins in distinct organelles through which mature MHC class II traffic.

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7. Appendix

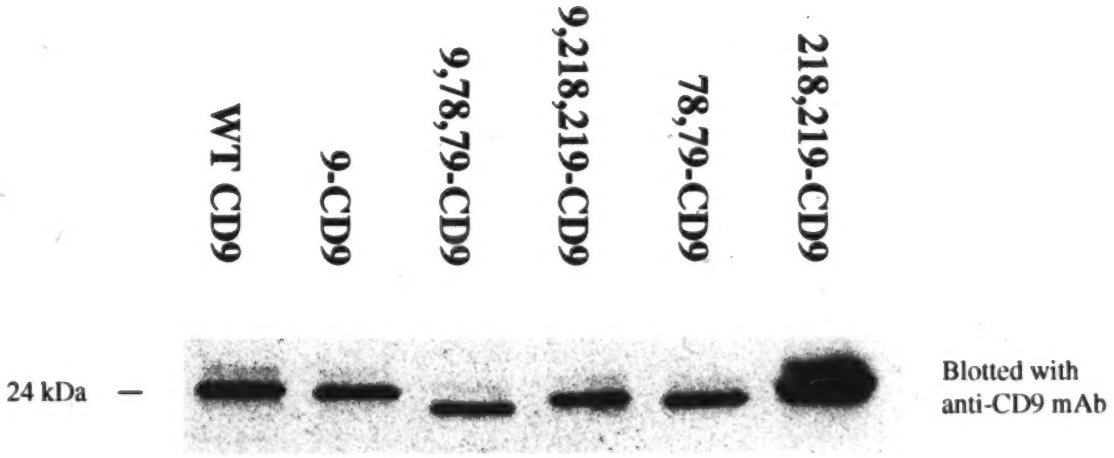

Figure 24. *SDS-PAGE mobility of immunoprecipitated CD9 with cysteine mutated to serine at positions 9; 78,79; 9,78,79; 218,219; 9,218,219.* Mutant cDNA's were checked by sequencing and stably expressed in Raji. Note several of the mutants exhibited increased electrophoretic mobility that could not be accounted for by loss of palmitate. The elevated expression of the 218,219 mutant is a stable phenotype that is reversed by co-mutation of cysteine at position 9. The minor N glycosylated isomer (26 kDa) is expressed in all the mutants implying normal trans-Golgi processing. Thus we have successfully produced stable CD9 cysteine mutants that with the exception of the 218,219 mutant are expressed at comparable and physiological levels.

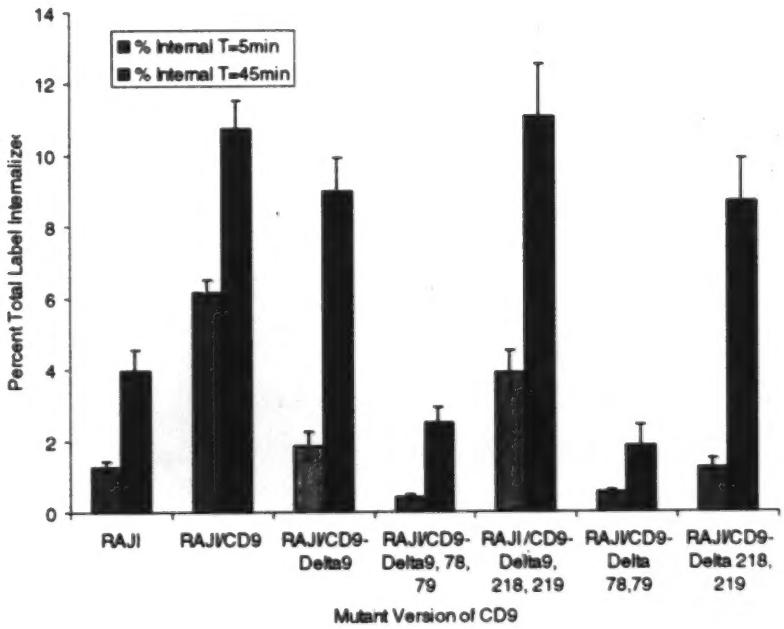

Figure 25. *Effect of cysteine to serine mutation at potential palmitoylation sites in CD9 on the CD9-enhancement of endocytosis of antibody ligated MHC class II in Raji /CD9.* MHC class II internalization is followed by 4-dimensional confocal microscopy. Cells are labeled by incubation on ice with 10 μg/mL of anti-MHC class II mAb 7H.3-Alexa 594. Cells are washed in two volumes of ice-cold culture supernatant followed by re-suspension in 2.0 mL of the same at 37°C in 35mm coverglass-bottomed petri dishes. Internalization is analyzed by 4-dimensional confocal imaging as described in Materials and Methods.

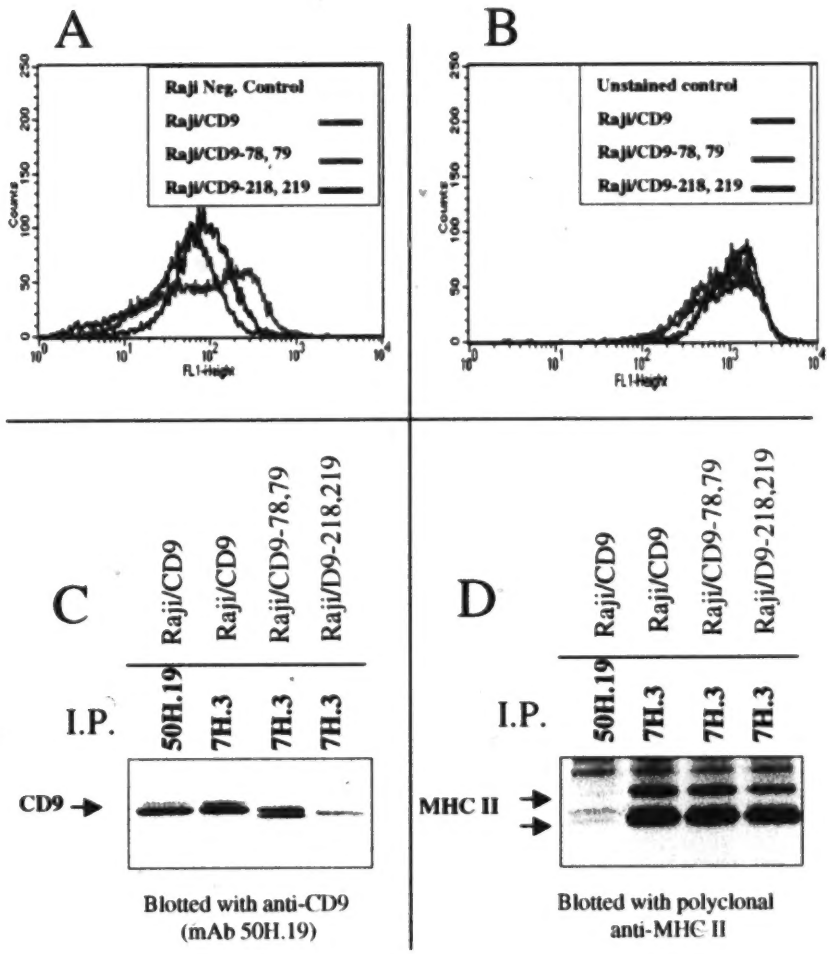

Figure 26. *Co-immunoprecipitation of Palmitoylation site mutated versions of CD9 with anti-MHC class II directed against surface MHC in a 1% CHAPS detergent lysate.* Flow cytometric analysis of surface expressed CD9 (A) and MHC class II (B) in Raji/CD9 transfectant cells carrying mutated versions of CD9. Cells are prepared by incubation with 10 µg/mL directly conjugated anti-CD9 mAb 50H.19-Alexa 488 or MHC class II mAb 7H.3-Alexa 488 for 45 min at 4°C. Cells are washed three times in RPMI 1640 pH 7.4 prior to analysis by flow cytometry. Co-immunoprecipitation of surface CD9 with anti-MHC class II (C). Cells are pretreated with 10µg/mL anti-MHC class II (7H.3) for 45 min at 4°C to specifically bind surface class II. Cells are then washed three times in PBS poH7.4 prior to lysis in 1% CHAPS lysis buffer to remove unbound anti-MHC class II. Bound MHC class II complexes are recovered from a cleared cell lysate by incubation with 30 µL protein A sepharose beads for 90 min at 4°C.

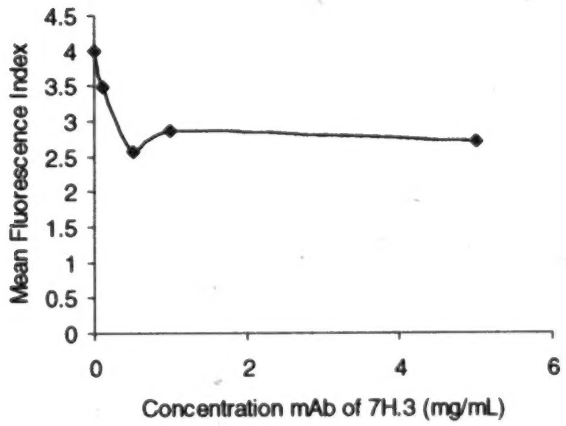

Figure 27. *Cortical F-actin Staining as A function of MHC class II crosslinking.* Raji cells were incubated in the presence of increasing concentrations of anti-MHC class II mAb 7H.3 for 60 min on ice. Following antibody ligation cells were treated with crosslinking rabbit anti-mouse polyclonal antibodies at 5 mg/mL for 30 min at 37°C. Following crosslinking cells were fixed on ice for 10 min in 2% PFA then stained with 1 μM phalloidin-FITC for 60 min on ice. Cells were then resuspended in CSB/BSA for analysis by flow cytometry to determine the mean fluorescence as an indicator of total cortical actin staining.